이학박사 학위논문

The heterotrophic dinoflagellates *Oxyrrhis marina* and *Oxyrrhis maritima* in Korean waters : taxonomy, physiology, distribution, and production of useful materials

한국연안의 종속영양성 와편모류 *Oxyrrhis marina*와 *Oxyrrhis maritima*의 분류, 생태, 분포 및 유용물질 생산에 대한 연구

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서울대학교 대학원

지구환경과학부 해양학전공

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지도교수 정 해 진

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Abstract

The heterotrophic dinoflagellates *Oxyrrhis marina* and *Oxyrrhis maritima* in Korean waters : taxonomy, physiology, distribution, and production of useful materials

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The genus *Oxyrrhis* is a heterotrophic dinoflagellate (HTD) which is often found in diverse marine environments such as coastal waters, tidal pools, and salterns. *Oxyrrhis* spp. have received much attention owing to their ecological and industrial importance. They play diverse roles such as prey for other protists or metazoans and predators for phytoplankton and bacteria in marine ecosystems. Furthermore, they produce several useful materials such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and anti-inflammatory materials.

For a long time, *Oxyrrhis marina* was considered a single species in the genus *Oxyrrhis,* after *Oxyrrhis marina, O. maritima, O. phaeocysticola,* and *O. tentaculifera* had been merged to *O. marina*. However, in 2011, *O. maritima* was separated from *O. marina* due to considerable difference in their genetic characterizations, although morphological difference between these two species was not significant. Although morphological and genetic characterizations are still important proxy of genomic characterizations, eco-physiological characterizations of these two species may provide a clue in solving the question whether they are the same or different species. To the best of our knowledges, many studies on eco-physiological characterizations of *O. marina,* but no studies on those of *O. maritima* have been reported until date. Therefore, I investigated the taxonomy and eco-physiology of *O. marina* and *O. maritima* using four *O. marina* strains from the coastal waters off Gunsan, Masan, Shiwha, and Karorim, Korea and two *O. maritima* strains were isolated from littoral tidepool waters in Jeju Island, Korea.

Difference among the sequences of the small subunit (SSU) rDNA of the four strains of *O. marina* was 0.3-3.5%, whereas that between two strains of *O. maritima* was only 0.3%. However, the difference between the sequences of SSU rDNA of *O. marina* and *O. maritima* was 17.3-18.1%. In the phylogenetic trees based on the SSU rDNA of dinoflagellates, the clade of all the four strains of *O. marina* was clearly divergent from that of the two strains of *O. maritima.* Furthermore, the *O. marina* strains belonged to the Lineage I (Clade 1 and 2), whereas the *O. maritima* strains belonged to the Lineage II (Clade 4). Thus, the results of this study suggest that *O. marina* and *O. maritima* are different species. Moreover, I found that the Korean strains of *O. marina* were splitted to two clades.

In the present study, I measured the growth rates of *O. marin*a and *O. maritima* under various water temperatures (5-36 ℃), salinities (2-90), and light intensities (0-100 μE $m^{-2} \cdot s^{-1}$). The strains of *O. maritima* grew at the temperatures of > 35 ℃, but *O. marina* did not grow. Furthermore, the optimal temperature supporting the maximum growth rate of *O. marina* was 25 ℃, while that of *O. maritima* was 30 ℃. Moreover, *O. marina* did not survive at a salinity of < 4, while *O. maritima* survived at a salinity of 2. In addition, at a salinity of 50, the growth rate of *O. maritima* (0.67 d^{-1}) was higher than that of *O. marina* (0.58 d^{-1}). Thus, *O. maritima* has wider ranges of temperature and salinity than for *O. marina.* Thus, theses two *Oxyrrhis* species may have different eco-physiological characterizations.

Using direct counting, real-time polymerase chain reaction (qPCR), and digital PCR (dPCR), I measured the abundances of *O. marina* and *O. maritima* in the waters collected from the 29 stations along the Korean coasts in January, March, May, July, August, October, and December in 2016 and also from tide-pools and salterns located in Taean and Jeju Island in June and September in 2016. In the dPCR method, *O. maritima* (177 ± 9.2 copies per cell) has copy numbers greater than *O. marina* (148 ± 6.9 copies per cell), even though *O. maritima* cell size is smaller than that of *O. marina.* In the qPCR and dPCR methods, the abundance of *O. marina* was rarely detected in Korean coastal waters, whereas that of *O. maritima* was frequently detected in southern Korea coastal waters in 2016. In addition, two species had much higher abundances in the tide pool or saltern (highest abundances : *O. marina,* 7,490 cells ml^{-1}; *O. maritima,* 3,700 cells ml^{-1}) than that in the coastal waters (highest abundances : *O. marina,* 39 cells ml^{-1}; *O. maritima,* 3 cells ml^{-1}). Therefore, the abundances

of *O. marina* and *O. maritima* were markedly affected by their habitats.

Moreover, I investigated new compounds from the massive culture of *O. marina*. *O. marina* can grow fast and is known to produce high amounts of useful lipids, such as docosahexaenoic acid (DHA). I isolated two new compounds, a trioxilin and a sulfoquinovosyl diacylglycerol (SQDG) from a massive culture of *O. marina,* which was cultivated by feeding on dried yeast. The complete structures of these compounds were determined using nuclear magnetic resonance (NMR) spectroscopy and chemical reactions. The trioxilin was identified as (4Z,8E,13Z,16Z,19Z)-7(S),10(S),11(S)-trihydroxydocosapentaenoic acid, and the SQDG was identified as (2S)-1-O-hexadecanosy‐2‐O‐docosahexaenoyl‐3-O-(6-sulfo-α-d-quinovopyranosyl)-glycerol by a combination of NMR spectra, mass, and chemical analyses. These two compounds were associated with DHA, which is a major component of *O. marina*. The two isolated compounds showed significant anti-inhibitory activity in lipopolysaccharide-induced RAW264.7 cells. The second compound showed no cytotoxicity against hepatocarcinoma (HepG2), neuroblastoma (Neuro-2a), and colon cancer (HCT-116) cells, while weak cytotoxicity of the first compound 1 against Neuro-2a cells was observed.

Furthermore, I investigated a new massive culture method which can produce high levels of DHA from *O. marina*. *O. marina* contains high levels of DHA when fed on diverse algal prey. However, large-scale culturing of algal prey species is not easy and requires a large amount of budget. Therefore, a more easily cultivable and low-cost prey is required. I found out that dried yeast was a strong candidate for an alternative prey and explored the fatty acid composition and DHA production of *O. marina*

fed on dried yeast. In addition, I compared these results with those of *O. marina* fed on two algal prey species: the phototrophic dinoflagellate *Amphidinium carterae* and chlorophyte *Chlorella* sp. powder. *O. marina* fed on dried yeast, which does not contain DHA, produced the high levels of DHA, in as those fed on DHA-containing *A. carterae.* This indicates that *O. marina* is likely to produce DHA regardless of the prey items. Furthermore, the DHA content (and portion of total fatty acid methyl esters) of *O. marina* satiated with dried yeast was 52.40 pg per cell and 25.9%), respectively. These values were considerably greater than those of *O. marina* fed on other algal prey species. Therefore, dried yeast is a more easily obtainable and cost-effective prey than is conventional algal prey for use in the production of DHA by *O. marina.*

In summary, I established clonal cultures of six different strains of *Oxyrrhis* species and identified them as *Oxyrrhis marina* and *O. maritima* based on morphological and molecular taxonomic analyses. In addition, I investigated their ecological and physiological response to diverse environmental parameters and distribution in Korean waters based on their taxonomic characteristics. Moreover, I investigated useful new compounds of *O. marina* and developed a new cost-effective mass culture method for this species. The finding of this study will contribute to the improvement of our understanding of the role of *Oxyrrhis* species in marine waters and commercialize useful materials from algal species.

Keywords : Heterotrophic dinoflagellates, *Oxyrrhis*, taxonomy、eco-physiology, abundance, useful new compounds, DHA production

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Chapter 1. Introduction

The heterotrophic dinoflagellates (HTDs) are ubiquitous protists and often abundant in marine environments (Jacobson 1987; Lessard 1984, 1991; Jeong et al., 1999, 2010; Wolny et al. 2015; Ok et al., 2017). The HTDs are known to feed on diverse prey items including bacteria (Jeong et al. 2008), picoeukaryotes (Lee 2006), nanoflagellates (Jeong et al. 2007b), diatoms (Jacobson and Anderson 1986; Menden-Deuer et al. 2005; Yoo et al. 2009), other dinoflagellates (Adolf et al. 2007; Tillmann 2004), heterotrophic protists (Hansen 1991; Bockstahler and Coats 1993) and metazoans owing to their diverse feeding mechanisms (Jeong 1999; Jeong et al. 2008, 2010). They are in turn ingested by many kinds of predators (Jeong 1999; Jeong et al. 2010). Thus, the roles of the HTDs in marine planktonic food webs are very diverse (Fig. 1-1). relationships in species level is important for understanding structures and functions of the marine ecosystem. Therefore, I established and identified the speciesIdentifying the HTD species and investigating their prey-predator of *Oxyrrhis marina* and *O. maritima* in Korean waters in Chapter 2.

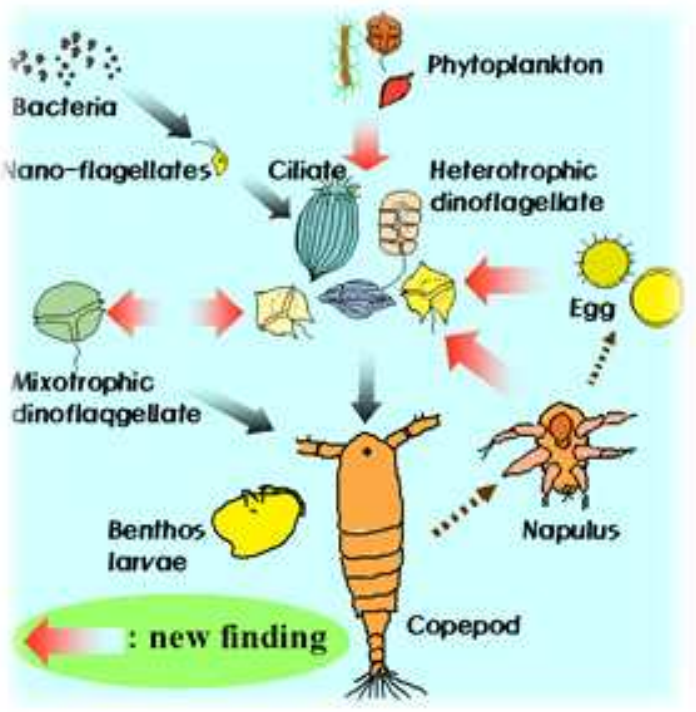

Fig. 1-1. Simplified scheme of the pelagic marine food web illustrating the processes.

The *Oxyrrhis* species has been often regarded as dinoflagellates (e.g. Kofoid & Swezy, 1921; Dodge, 1984; Sournia, 1986) but explicitly excluded from the group in other classification schemes (Fensome et al., 1993), because it has a number of characters that are very different from those of general dinoflagellates. The general morphology of dinoflagellates is unicellular, with two dissimilar flagella arising from the ventral cell side (Dodge 1971; Taylor 2006; Lowe et al. 2010, 2011b). Most of the dinoflagellates either have a longitudinal and a transverse flagellum, emerging from the sulcus and the cingulum, respectively, or have both flagella growing from the apical area (Prorocentrales) (Fig. 1-2, a). In

contrast, the genus *Oxyrrhis* dinoflgellate somewhat unusual dinoflagellate. Both flagella of *Oxyrrhis* grow from the ventral side (Fig. 1-2, b). Furthermore, the flagella of *Oxyrrhis* possess a row of complex mastigonemes but lack a broad striated strand on the transverse flagellum, and both the transverse and longitudinal flagella are covered with scales (Clarke 1972, 1976; Cachon 1988). Other differences between *Oxyrrhis* and the general morphology of dinoflagellates are the lack of a girdle, a sulcus or pusules (some dinoflagellates have secondarily lost the girdle and/or sulcus; Fensome et al., 1993) and the presence of putative histone proteins in *Oxyrrhis* (Kato et al., 1997). Histones are absent from in the nuclei of most dinoflagellates (the order Syndiniales could be an exception: Ris & Kubai, 1974; Sala-Rovira et al., 1991).

O. marina is the type species of the genus *Oxyrrhis*. The features mentioned above make this species cytologically and genetically closer to a typical eukaryote than a typical dinoflagellate. The phylogenetic position of *O. marina* is controversial. Some of the morphological and cytological studies support its basal position in the dinoflagellate lineage (Cachon et al., 1979; Kato et al., 2000; Montagnes et al., 2011), whereas others infer a highly derived position within the order of Gonyaulacales (Kofoid and Swezy 1921; Saldarriaga 2003; Cavalier-Smith and Chao, 2004; Guo et al., 2013). However, with increasing molecular data on dinoflagellates, such as the phylogenies based on various genes, mitochondrial genome structure, RNA editing, and trans-splicing of nucleus-encoded mRNA (Lenaers et al., 1991; Leander 2004; Slamovits et al., 2007; Zhang and Lin, 2008), the majority of recent studies supports *O. marina* as an ancestral dinoflagellate lineage (Hoppenrath and Leander 2010).

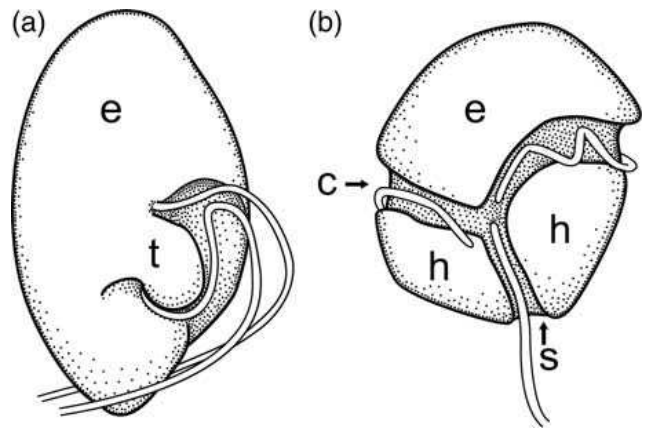

Fig. 1-3. Schematic illustrations of (a) *Oxyrrhis marina* (left) and (b) a generalized general dinoflagellates indicating (right): the epicone (e), the hypocone (h), the tentacle (t)/ventral bulge, the cingulum (c) and the sulcus (s). (Lowe et al., 2011b).

Morphological studies have raised disputes on whether the genus *Oxyrrhis* contains multiple species (*O. marina, O. maritima* van Meel 1958, *O. phaeocysticola* Scherffel 1900 and *O. tentaculifera* Conrad 1939) or only one species (*O. marin*a) (Scheffel 1900; Kofoid and Swezy 1921; Conrad 1939; Van Meel 1958; Dodge 1982; Cvalier-Smith and Chao 2004; Lowe et al., 2011b). Recent molecular phylogenetic studies favor the notion that two sibling species exist in this genus. Cavalier-Smith and Chao (2004) suggested that *Oxyrrhis* consists of at least two species based on sequence variations in the small subunit ribosomal RNA gene (SSU rDNA). Recently, Lowe et al. (2011) suggested that the genus consists of two species *O. marina* and *O. maritima.*

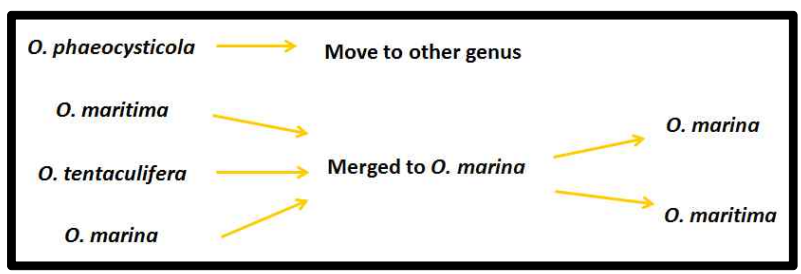

Fig. 1-4. History of the taxonomy of the genus *Oxyrrhis*

The genus *Oxyrrhis* is a widespread, free-living, and ecologically important HTD (Capriulo 1990; Slamovits and Keeling 2008; Lowe et al., 2011a; Watts et al., 2011). *Oxyrrhis marina* is commonly found in marine and waters near the shore, including rock pools, estuaries and marshes **(**Kofoid & Swezy, 1921; Dodge, 1984; Sournia, 1986**).** It is an important model organism for a broad range of ecological (Tillmann 1998 and 2003; Suttle 2007; Mariani et al., 2008; Boakes et al., 2011; Roberts et al., 2011; Yang et al., 2011), biogeographic (Lowe et al., 2005; Montagnes et al., 2011; Hartz et al., 2011), and evolutionary studies (Lowe et al., 2011a; Montagnes et al., 2011; Yang et al., 2011). Despite the increasing number of studies on this organism, the existing data is scattered, remaining to be synthesized (Guo et al., 2013).

Oxyrrhis marina is unlikely to be a single species, and there are strain-specific in eco-physiological responses (Lowe et al., 2005a, 2010). For instance, the growth response of *O. marina* strains differs based on responses to: salinity (Lowe et al., 2005a), prey concentration and type, and temperature (Montagnes et al., 2011). However, such strain-specific responses are far from unique to *O. marina*; e.g., similar strain-specific differences occur in a model freshwater ciliate, Urotrichia (Weisse and Montagnes, 1998). Thus, modelers must be aware of these differences and consider them when interpreting results. In fact, as strain-specific are becoming importance topicals in ecological research, this “problem” can converted into asset, and modelers will undoubtedly begin to use the responses of the various strains to examine potential strain‐succession, as we are at present doing (Yang et al. 2011). I compared growth rates based on the distinction of the species into *O. marina* and *O. maritima* in Chapter 3.

HTDs are known to feed on variety of prey species, including harmful algal species such as *Prorocentrum minimum, Karlodinium veneficum, Ostreopsis ovata, Cochlodinium polykrikoides,* and *Pfiesteria piscicida* (Kim and Jeong, 2004; Adolf et al., 2007; Yoo et al., 2015**).** Some HTDs may sometimes have a considerable grazing impact on populations of marine harmful algal species the grazing impac (Jeong et al., 2010). Therefore, studying the distribution and abundance of each species and the predator-prey relationships in the environmental water is very important for understanding and predicting effects on the marine ecosystem. Accurate abundance and biomass measurements are essential steps in determining the role of plankton in the microbial food web (Zarauz and Xabier 2008). Therefore, I measured the distributions and abundances of *O. marina* and *O. maritima* in the coastal waters, tide-pools, and salterns of Korea by direct counting, real-time PCR, and digital PCR in Chapter 4.

Marine plankton has been recognized as rich sources of proteins, lipids, vitamins, pigments, and other nutrients (Spolaore et al. 2006). In addition, they produce high-value chemicals including carotenoids (Borowitzka 2010), long-chain polyunsaturated fatty acids (Ratledge 2004; Mendes et al. 2009), phycobilins (Samarakoon et al., 2013), and compounds with potential structures for novel drugs. Therefore, there have been studies for exploration of marine bio-resources which is an indefinite challenge for bio-mining researchers. Recently, the trends of efforts on commercialization of marine plankton are increasing globally. Increasing numbers of natural products were investigated on a broad spectrum of pharmacological activity. Several protists contain high levels of eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) (Tang and Taal 2005, Burja et al. 2006, Veloza et al. 2006, Liu

et al. 2014). Therefore, I designed a novel and effective batch culture system to mass cultivate (for Chapter 5 & Chapter 6) of the *O. marina,* which contains useful new compounds and high levels of DHA.

Outline of this thesis

Oxyrrhis marina is an ideal candidate organism for the experimental study and modeling of the natural and theoretical population dynamics of protozoan predators. It is easy to find, isolate, maintain in culture and manipulate in the laboratory, and it has been maintained in culture for over 50 years in a number of culture collections (Montagnes et al., 2011). Therefore, *O. marina* is a very important 'model species' in HTDs. Therefore, many scientists have studied it for a long time due to the following reasons; the species is easily found in the natural waters, ponds, brackish, inter-tide ponds, and even salton pond; it is can be cultured easily in the laboratory because it can survive under broad physical conditions (e.g., light, salinity, and temperature) and utilize diverse prey items (e.g., bacteria, heterotrophc nanoflagellates, dinoflagellates, and raphidophytes).

In Chapter 2, I established cultures of six strains of *Oxyrrhis* (*O. marina* and *O. maritima*) after isolation from six different locations in the Korean environmental water samples. I analyzed the morphological features, phylogeny, and rDNA based on the six strains of *Oxyrrhis* spp.

In Chapter 3, I compared the specific growth rate of *O. marina* and *O. maritima* under various conditions such as temperature, salinity, and light intensity. In particular, I analyzed the upper limit, lower limit, and optimal range of each environmental factor to test whether *O. marina* and *O. maritima* have different eco-physiology, reflecting their habitats.

In Chapter 4, I established an efficient method for the quantification and abundance for monitoring of *O. marina* and *O. maritima* using the real-time PCR (qPCR) assay and digital PCR (dPCR). Based on the standard curve of qPCR and standard gene size revealed by dPCR, I applied the novel techniques to environmental samples. Thus, the temporal variations in abundance of *O. marina* and *O. maritima* measured from Korean waters in 2016.

In chapter 5, I described the isolation, process of determination of the structure of two new compounds, and biological activity from mass cultures of *O. marina*. Intensive research would be required to assure high levels of productivity for matching commercial-scale requirements.

In Chapter 6, I compared the fatty acid composition and DHA content of *O. marina* fed on dried yeast are compared to those of *O. marina* fed on algal prey (*A. carterae* or dried powders of *Chlorella* sp.). In addition, I also explored *O. marina* fed dried yeast, *A. carterae, Chlorella* sp. powder at satiated and starved conditions, respectively. Thus, I suggest a new method for cultivating *O. marina* with high DHA levels using low-cost prey that can be easily obtainable.

The results of this thesis provide a basis on understanding the diverse ecological roles of *Oxyrrhis* spp. at the species-level in Korean waters. Furthermore, the development of an effective *O. marina* mass cultivation method for producing marine utilizes valuable bio-resources.

Chapter 1	**Overall introduction (Backgrounds)**
Chapter 2	**Taxonomy of 6 strains of *Oxyrrhis* spp. using rDNA, gene diversity, phylogenetic, and morphological features**
Chapter 3	**Physiology of *Oxyrrhis* spp. : temperature, salinity, and light intensity.**
Chapter 4	**Distribution and abundance of target species in Korean waters** (coastal waters, salterns, and tide-pools)
Chapter 5	**Two novel compounds from *O. marina***
Chapter 6	**DHA content of *O. marina* fed on dried yeast: compared with algal prey**
Chapter 7	**Overall Conclusion**

Fig. 1-4. The frame of Ph.D. thesis

Chapter 2. Taxonomy of *Oxyrrhis marina* and *O. maritima* isolated from Korean coastal waters, saltern, and tide-pool using rDNA, gene diversity, phylogenetic, and morphological features

2-1. Introduction

HTDs are ubiquitous protists in marine environments and play diverse ecological roles in marine plankton food webs (Jeong 1999; Lessard 1991; Sherr and Sherr 2007; Jeong et al. 2010); they feed on a diverse array of prey species and in turn serve as prey for diverse predators (Jeong 1999; Menden-Deuer et al. 2005; Park et al. 2007). Each HTD plays different ecological roles in the marine food web. Therefore, taxonomy and eco-physiology of HTDs should be revealed at the species level.

The genus of *Oxyrrhis* is a HTD commonly found in marine and brackish near-shore waters, tide-pools, and slaternsalterns. Furthermore, these species are easy culturing to culture and maintaining in the laboratory. Therefore,. *Oxyrrhis* is a widespread, free-living, and ecologically important HTD (Slamovits and Keeling 2008; Lowe et al., 2011; Watts et al., 2011). In addition, It it is also an important model organism for a broad range of ecological (Tillmann 1998 and 2003; Suttle 2007; Mariani et al., 2008; Boakes et al., 2011; Roberts et al., 2011; Yang et al., 2011), biogeographic (Lowe et al., 2005; Montagnes et al., 2011; Hartz et al., 2011), and

evolutionary studies (Lowe et al., 2011; Montagnes et al., 2011; Yang et al., 2011).

Early studies recognized the genus of *Oxyrrhis* to be a dinoflgellate, though a somewhat unusual one. This genus displays many characteristics that differ from those in other dinoflagellates. The majority of dinoflagellates either have a longitudinal and a transverse flagellum, emerging from the sulcus and the cingulum, respectively, or both flagella growing from the apical area (Prorocentrales). In contrast, *Oxyrrhis*'s both flagella grow from the ventral side. Furthermore, the flagella in *Oxyrrhis* possess a row of complex mastigonemes while lack a broad striated strand on the transverse flagellum, and both transverse and longitudinal flagella are covered with scales. The phylogenetic position of *O. marina* is controversial. Some of the morphological and cytological studies support its basal position in the dinoflagellate lineage, while others infer a highly derived position within the order of Gonyaulacales the majority of recent studies supports *O. marina* as an ancestral dinoflagellate lineage (Guo et al., 2013).

Morphological studies have raised disputes on whether the genus *Oxyrrhis* contains multiple species (*O. marina, O. maritima* van Meel 1969, *O. phaeocysticola* Scherffel 1900 and *O. tentaculifera* Conrad 1939) or only one species (*O. marin*a) (Scheffel 1900; Kofoid and Swezy 1921; Conrad 1939; Van Meel 1958; Dodge 1982; Cvalier-Smith and Chao 2004; Lowe et al., 2011b). Recently molecular phylogenetic studies favor the notion that two sibling species exist in this genus. Lowe et al. (2011b) suggested this genus consists of two species, *O. marina* and *O. maritima* and each species consists of two clades.

Despite the increasing number of studies on *O. marina*, the existing data is scattered, remaining to be synthesized (Guo et al., 2013). Ecological, physiological, and genetical studies have been conducted without accurate identification of species. Here, I established the four strains of *O. marina* and two strains of *O. maritima* species by isolation and culturing from six difference locations in the Korean environmental water samples. I analyzed the morphological features, phylogney, and rDNA based on the 6 strains of *Oxyrrhis* spp.

I provided information on the taxonomy of the strains of this genus collected from the Korean waters in species level. It might help us to understand the structure and function of the marine ecosystems, and it may also facilitate ecological, physiological, and genetic studies owing to accurate species identification.

2-2. Materials and methods

2.2.1. Collection, isolation, and culturing of *Oxyrrhis* spp.

Water samples were collected from the surface at each station with a water sampling bucket from littoral tide-pools in the Jeju Island and coastal waters in Korea (Shiwha, Gunsan, Masan, Jeju Island) (Table 2-1; Fig. 2-2). Plankton samples were collected from surface waters of six different stations off Shiwha (37°18′00.1″N, 126°40′19.5″E), Karorim (36°45″19.1″N, 126°11′28.0″E), Gunsan (35°56′20.1″N, 126°31′50.5″E), Masan (35°11′41.2″N, 128°34′23.3″E), and Jeju Island Jeju-Anduck (33°12′47.7″N, 126°17′40.6″E) and Jeju-Hamduck (33°32′33.6″N, 126°40′10.7″E) from May 2001 to May 2013. Water temperature and salinity measured during sampling for *O. marina* were 13.5 – 19.7°C and 27.7 - 33, respectively, but no physical information was available in case of *O. maritima.*

The sample was filtered through a 154-μm Nitex mesh screen and placed in six-well tissue culture plates. A clonal culture was established following two serial single-cell isolations, and the dried yeast (*Saccharomyces cerevisiae*) obtained from Red Star (Lesaffre Yeast Corporation, Milwaukee, WI, USA) or *Amphidinium carterae* was provided as food. As the volume of culture increased, cells were transferred to 32-, 250-, and 500-mL polycarbonate (PC) bottles. The bottles were placed on a shelf at 20°C under continuous illumination of 10 μmol m^{-2} s^{-1} provided by a cool-white fluorescent light source. When cultures became dense, approximately every 2–3 d, they were transferred to new 500-mL PC bottles containing dried yeast or *A. carterae* as prey.

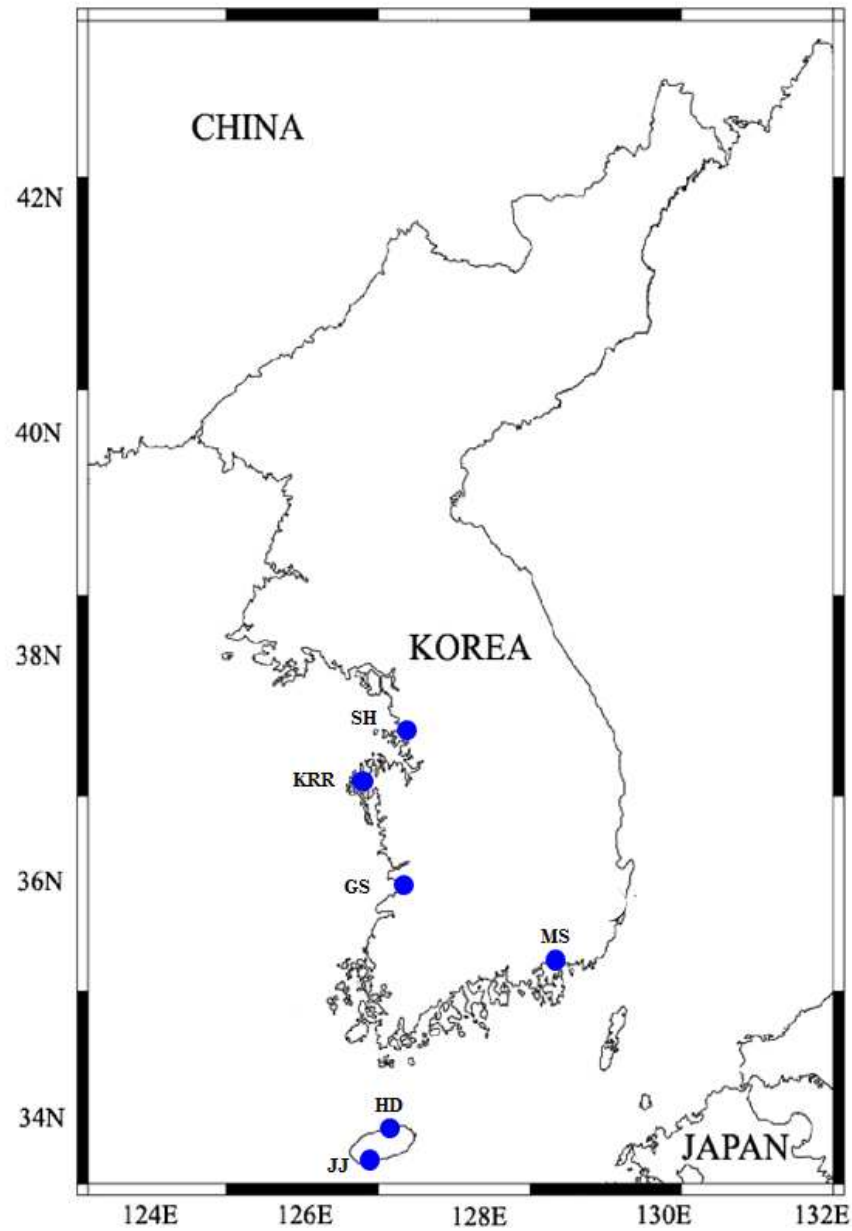

Fig. 2-1. Maps of the sampling stations of *Oxyrrhis marina* [SH (Shiwha), KRR (Karorim), GS (Gunsan), MS (Masan), Korea] and *O. maritima* [JJ (Jeju-Anduck) and HD (Hamduck), which is located in Jeju Island, Korea]

Table 2-1. Locations, water temperature, salinity, and habitats where *Oxyrrhis* spp. were found in the coastal waters of Korea during the study periods

Location	Date	GPS	Temperature (℃)	Salinity	Habitats	Strain Name
Shiwha (SH)	2011.11	37°18'00.1"N 126°40'19.5"E	19.7	31	Coastal	OMSH_201111
Karorim (KRR)	2010.05	36°45'19.1"N 126°11'28.0"E	19.5	33	Saltern	OMKRR_201005
Gunsan (GS)	2001.05	35°56'20.1"N 126°31'50.5"E	16	27.7	Coastal	OMGS_200105
Masan (MS)	2010.04	35°11'41.2"N 128°34'23.3"E	13.5	29.8	Coastal	OMMS_201004
Jeju Island (JJ)	2013.05	33°12'47.7"N 126°17'40.6"E	No information		Tide pool	OMJJ_201305
Jeju Island (HD)	2013.05	33°32'33.6"N 126°40'10.7"E			Tide pool	OMHD_201305

2.2.2. Microscopic observations

The morphology of *Oxyrrhis marina* and *O. maritima* were examined using light microscopy. The length and width of live vegetative flagellated cells were measured using an image analysis system for processing images taken with a compound microscope (BX53 microscope, Olympus, Japan).

2.2.3. DNA extraction, PCR amplification, and sequencing

Three to five cells of *Oxyrrhis marina* and *O. maritima* from a clonal culture were transferred to a 0.2-mL PCR tube containing 38.75 μL distilled water. To lyse the cell membranes before PCR analysis, the tube was frozen at −72°C for 1–3 min and then thawed. The final mixture (50 μL) was vortexed and PCR reaction was performed as follows: a thermal cycler (Eppendorf AG, Mastercycler® ep, model 5341, Hamburg, Germany) was used for the PCR reactions. The final concentrations of PCR products were: 5 μL of 10X F-Star Taq buffer, 1 μL of 10 mM of dNTP mix, 0.25 μL of 5 U/μL BioFACT™ F-Star Taq DNA polymerase (BioFACT Co., Ltd., Daejeon, Korea), 0.02 μM of each primer, for a final volume of 50 μL. The following primer pairs were used for amplification of SSU rDNA segments, ITS region, and LSU rDNA sequences: EukA (5′-CTG GTT GAT CCT GCC AG-3′; Medlin et al. 1988) and G18R (5′-GCA TCA CAG ACC TGT TAT TG-3′; Litaker et al. 2003); 570F (5′-GTA ATT CCA GCT CCA ATA GC-3′; Moorthi et al. 2006) and EukB (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′; Medlin et al. 1988); ITSF2 (5′-TAC GTC CCT GCC CTT TGT AC-3′; Litaker et al. 2003) and LSU500R (5′-CCC TCA TGG TAC TTG TTT GC-3′; Litaker et al. 2003); and D1RF (5′-ACC CGC TGA ATT TAA GCA-3′; Scholin et al.

1994), and 1483R (5′-GCT ACT ACC AAG ATC TGC-3′; Daugbjerg et al. 2000). The PCR conditions were as follows: one activation step at 95°C for 2 min; followed by 40 cycles at 95°C for 20 s, the selected annealing temperature for 40 s, and 72°C for 1 min; a final elongation step at 72°C for 5 min. The AT was adjusted depending on the primers used according to the manufacturer's instructions. Positive and negative controls were used for all amplification reactions. The purity of the amplicons was checked by eletrophoresis of 5 μL of PCR products mixed with 1 μL of Red fluorescent reagent (Redstar, Daejeon, Korea) using a 0.9% agarose gel at 80 V and observing it under a UV lamp to ensure that a single product was formed. Products containing a single band were then purified using an AccuPrep® PCR purification kit (Bioneer Corp., Daejeon, Korea) according to the manufacturer's instructions. The purified amplicon was sent to the Biomedic Corporation (Gyeonggi, Korea) for sequencing using ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Each portion of the target DNA was independently sequenced three times in both directions using primer pairs identical to those used for DNA amplification. These sequences were aligned using the ContigExpress alignment program (InforMax, Frederick, MD, USA) to remove low-quality regions and to assemble the individual sequence reads.

2.2.4. Phylogenetic analysis

Phylogenetic analyses were performed based on alignments of partial 3′ end SSU and ITS1-5.8S-ITS2 sequences. Analyses included sequences obtained from GenBank and the present study (Table 2-2; Table 2-3) Multiple sequences were aligned automatically using MEGA4′s native implementation of ClustalW (Tamura et al. 2007), and further aligned

manually for refinement of the alignments. Maximum-likelihood (ML) analyses were performed using the RAxML 7.0.3 program (Stamatakis 2006) with default GTR + G model. Tree likelihoods were estimated using a heuristic search with 100 random additional sequence replicates and tree bisection and reconnection branch swapping. ML bootstrapping with 1,000 replications was also conducted. Bayesian analyses were performed using MrBayes v.3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) with the GTR + G model to determine the best available model for the data for each region.

Table 2-2. List of species used in constructing SSU ribosomal (r) DNA phylogenetic trees. Genbank accession numbers are listed to the right of each species.

Species	Strain no.	Accession number	Nm sizes(bp)	origin
Oxyrrhis marina KRR			1692	This Study
Oxyrrhis marina SH			1690	
Oxyrrhis marina MS			1696	
Oxyrrhis marina JJ			1671	
Oxyrrhis marina GS			1675	
Oxyrrhis marina HD			1672	
Oxyrrhis sp.	WHOI_LI1-5-6	AF330215.1	1759	UK
Oxyrrhis marina		AF482425.1	1715	CAN
Oxyrrhis marina		AB033717.1	1749	JPN
Fragilidium sp.	G02	FJ405356.1	1698	CHI
Pyrocystis sp.	CCAP1131/1	FR865628.1	1706	UK
Abedinium dasypus	FG257	GU355679.1	1212	FRG
Neoceratium.digitatum	FG231	FJ824910.1	1220	FRG
Ceratium longipes	ccmp1770	DQ388462.1	1709	USA
Prorocentrum sp.	IRTA002	FJ160591.1	1677	SPN
Blastodinium contortum		DQ317537.1	1799	DEN
Prorocentrum dentatum	CCMP1517	AY803742.1	1782	CHI
Oxyrrhis marina	CCAP1133/3	AY566418.1	1603	UK

Oxyrrhis marina		AF280077.1	1732	UK
Paramecium tetraurelia	hrd	AB252009.1	1745	JPN
Paramecium tetraurelia	51	AB252008.1	1745	JPN
Colpoda sp.	PRA-118	AY905498.1	1778	USA
Colpidium colpoda		EF070266.1	1624	CAN
Sarcocystis miescheriana	SmJY3	JX840466.1	1677	CHI
Sarcocystis sinensis	2CHRF50	JX679469.1	1843	ARG
Sarcocystis fusiformis		U03071.1	1878	JPN
Gonyaulax spinifera		AF052190.1	1795	MAL
Heterocapsa triquetra	MBIC11142	AB183670.1	1754	JPN
Prorocentrum micans	UTEX1003	EF492511.1	1795	USA
Blepharisma americanum		AM713182.1	1673	GER
Oxytricha sp.	JS-2012	JX893371.1	1769	IND
Oxytricha sp.	ML111	FN429124.1	1723	GER
Colpodella sp.	ATCC50594	AY142075.1	1730	CAN

Table 2-3. List of species used in constructing ITS1-5.8S-ITS2 phylogenetic trees. Genbank accession numbers are listed to the right of each species.

Species	Strain no.	Accession number	Nm sizes (bp)	origin
Oxyrrhis marina KRR			392	This Study
Oxyrrhis marina SH			406	
Oxyrrhis marina MS			406	
Oxyrrhis marina JJ			454	
Oxyrrhis marina GS			407	
Oxyrrhis marina HD			457	
Oxyrrhis marina	CCAP1133/3	AY566418.1	1603	UK
Oxyrrhis marina	CCAP1133/4	AY566417.1	1598	UK
Oxyrrhis marina	CCAP1133/5	AY566416.1	1598	UK
Paramecium tetraurelia		AB252045.1	513	JPN
Paramecium tetraurelia	51	AB252044.1	513	JPN
Gonyaulax spinifera		AF051832.1	510	MAL
Heterocapsa triquetra	NEPCC900	JQ972678.1	641	UK
Heterocapsa triquetra		AB084101.1	594	JPN
Prorocentrum micans	AC202	FJ823583.1	1755	CAN
Prorocentrum micans	CCAP1136-18	FJ823584.1	611	CAN
Oxytricha sp	LS38	AF508764.1	3627	USA
Toxoplasma gondii		JX456456.1	428	CHI
Symbiodinium microadriaticum	NEPCC411	FJ823605.1|	1790	CAN
Symbiodinium microadriaticum	CCMP2469	FJ823604.1	537	CAN

Oxyrrhis marina	StAndr	FJ853681.1	390	UK
Oxyrrhis marina	Giants	FJ853663.1	390	IRE
Oxyrrhis marina	Carror	FJ853659.1	390	IRE
Oxyrrhis marina	Cladda	FJ853660.1	390	IRE
Oxyrrhis marina	Aberys	FJ853657.1	390	UK
Oxyrrhis marina	Llandu	FJ853667.1	390	UK
Oxyrrhis marina	Faro	FJ853662.1	390	UK
Oxyrrhis marina	Mostei	FJ853670.1	390	POR
Oxyrrhis marina	Sao_Ro	FJ853679.1	390	POR
Oxyrrhis marina	Bahar	FJ853668.1	405	UK
Oxyrrhis marina	Naxos	FJ853674.1	394	GRE
Oxyrrhis marina	NaplesA	FJ853671.1	392	ITA
Oxyrrhis marina	NaplesB	FJ853672.1	393	UK
Oxyrrhis marina	NaplesC	FJ853673.1	390	ITA
Oxyrrhis marina	Okinaw	FJ853675.1	393	JPN
Oxyrrhis marina	CCMP1739	AY566413.1	432	USA
Oxyrrhis marina	CCMP605	AY566414.1	431	USA
Oxyrrhis marina	CCMP604	AY566415.1	431	USA
Oxyrrhis marina	CCMP1795	AY566411.1	431	USA
Oxyrrhis marina	CCMP1788	AY566412.1	431	UK
Oxyrrhis marina	Gunsan	FJ853666.1	390	KOR
Oxyrrhis marina	Porto	FJ853677.1	405	GRE
Oxyrrhis marina	Posith	FJ853678.1	405	GRE
Oxyrrhis marina	Sounio	FJ853680.1	394	GRE
Oxyrrhis marina	Isle	AY566410.1	430	UK
Oxyrrhis marina	Cromer	FJ853661.1	390	UK

2-3. Results

2.3.1 Morphology of *Oxyrrhis marina*

Under a light microscope (LM), the living cell were observed to be subovoid, asymmetrical posteriorly; girdle postmedial, not extending to dorsal surface; sulcus spreading posteroventrally; flagella midventral; tentacular lobe occurs between two flagella, dividing the broad undeveloped ventral sulcus. It appeared colorless but with pink pigmentation that is apparent in concentrated cultures. Therefore, this species called as “pink pig” because of large concentration of these organisms an colored the water red (Klein et al. 1986) (Fig. 2-2).

The length and width of living cells of starved *O. marina* (n = 30) ranged 16.0 – 31.2 μm (mean ± SD = 23.6 ± 4.2 μm) and 8.0 – 19.2 μm (13.8 ± 2.4 μm), respectively, whereas live cells fed with *Prorocentrum minimum* (n = 15) measured 15.2 – 33.7 μm (28.4 ± 4.64 μm) and 15.6 – 24.0 μm (18.9 ± 3.7 μm) in length and width, respectively. However, the ratio of the cell length to width of living cells starved for 5 d (mean ± SD = 1.74 ± 0.23; range = 1.2 – 2.2, n = 30) was larger than that of cells fed *P. minimum* (mean ± SD = 1.53 ± 0.17; range = 1.3 – 1.8, n = 15).

The mean equivalent spherical diameter (ESD, mean ± SD was measured using an electronic particle counter (Coulter Multisizer II; Coulter Corp., Miami, FL, USA). The ESD of *Oxyrrhis marina* (OMGS 200105) starved for 5d (n > 2,000) was 17.11 - 22.72 μm (19.85 ± 2.54 μm).

Fig. 2-2. Micrographs of *Oxyrrhis marina* (OMGS 2001) using light microscopy. Scale bars = 20 μm .

2.3.2 Morphology of *Oxyrrhis maritima*

Under LM, living cells were observed to be subovoid, posteriorly asymmetrical; girdle postmedial, not extending to dorsal surface; sulcus spreading posteroventrally; flagella midventral; tentacular lobe present between two flagella, dividing the broad undeveloped ventral sulcus. It appeared colorless but has pink pigmentation that is apparent in concentrated cultures (Fig. 2-3).

The length and width of living cells of *O. maritima* starved for 5 d (n=30) ranged 14.4 – 28.0 μm (mean ± SD = 20.6 ± 3.3 μm) and 9.6 – 15.2 μm (11.8 ± 1.9 μm), respectively, whereas live cells fed with *P. minimum* (n=15) measured 16.8 – 35.2 μm (27.6 ± 5.1 μm) and 11.2 – 24.7 μm (19.9 ± 3.6 μm) in length and width, respectively. However, the ratio of length to width of living cells starved for 5 d (mean ± SD = 1.74 ± 0.23; range = 1.2 – 2.2, n = 30) was larger than that of the cells of *O. marina* fed with *P. minimum* (mean ± SD = 1.53 ± 0.17; range = 1.3 – 1.8, n = 15). The ESD of *O. maritima* (OMJJ 201305) measured 11.4 - 15.1 μm (13.3 ± 1.5 μm), respectively. The ESD of *O. maritima* was approximately 45% smaller than that of *O. marina.*

Fig. 2-3. Micrographs of *Oxyrrhis maritima* (OMJJ 2015) using light microscopy. Scale bars = 20 μm.

2.3.3 DNA sequence analysis (genetic variability)

Sequences of SSU, ITS, and LSU rDNA were obtained from four strains of *Oxyrrhis marina* and two strains of *O. maritima*. The SSU, ITS 1 and 2, 5.8S, and LSU rDNA sequences of six strains of *Oxyrrhis* spp. from Gunsan (GS), Karorkim (KRR), Masan (MS), Shiwha (SH), Jeju Anduck (JJ), Jeju Hamduck (HD) comprised 2,882, 3,010, 3,033, 3,006, 3,051, and 3,038 nucleotides (Table 2-4).

Table 2-4. The number of the rDNA sequence from this study.

	Gunsan (GS)	Karorim (KRR)	Masan (MS)	Shiwha (SH)	Jeju JJ (JJ)	Jeju HD (HD)
Total (bp)	2,882	3,010	3,033	3,006	3,051	3,038
SSU	1,675	1,692	1,690	1,696	1,671	1,655
ITS1-5.8S-ITS2	407	392	406	406	454	457
LSU	800	926	937	904	926	926

I compared the sequences of *Oxyrrhis* spp. Sequence alignments were performed using the DNA Star suite of software. Sequence divergence was exceptionally high among some strains (Table 2-5). I compared the genetic divergence (%) of SSU (Small subunit), ITS1-5.8S-ITS2, and LSU (Large subunit) of six strains of *Oxyrrhis* spp.

When properly aligned, the SSU rDNA sequence of *O. maritima* (JJ) showed differed greatly from those of *O. marina* isolated from Gunsan, Karorim, Masan, and Shiwha. There was 215, 209, 205, and 208 bp difference (except Gap), and the similarity was 82.5%, 82.7%, 82.4% and 81.9%, respectively (corresponding to 17.3-18.1% dissimilarity). Within the ITS1-5.8S-ITS2 region, the result showed that there was 1-18bp difference and 4-22% dissimilarity. In addition, the LSU rDNA sequences of the Jeju1 strain of *O. marina* were of 19, 23, and 20 bp (i.e., 90.5%, 89.1%, and 89%), respectively. Moreover, 9.5-11% difference was found from the Gunsan, Karorim, Masan and Shiwha.

Difference among the sequences of SSU rDNA of the four strains of *O. marina* was 0.3-3.5%, whereas that between the two strains of *O. maritima* was only 0.3%. However, the difference between the sequences of SSU rDNA of *O. marina* and *O. maritima* was 17.3-18.1%. In the phylogenetic trees based on the SSU rDNA of dinoflagellates, the clade of all the four strains of *O. marina* was clearly divergent from that of two strains of *O. maritima.* Furthermore, the *O. marina* strains belonged to Lineage I (Clade 1 and 2), whereas the *O. maritima* strains belonged to the Lineage II (Clade 4).

Table 2-5. Comparison of the sequences of the small subunit (SSU), internal transcribed spacer (ITS1)-5.8S-ITS2, and large subunit (LSU) of *Oxyrrhis marina* (KRR, SH, MS, GS) and *O. maritima* (JJ & HD). The numbers are base pairs different from each other. The numbers in parenthesis are similarity (%) except gaps.

A. SSU of *Oxyrrhis* species.

	O. marina	*O. marina*	*O. marina*	*O. maritima*	*O. marina*	*O. maritima*
	KRR	SH	MS	JJ	GS	HD
KRR	-					
SH	53 (96.5%)					
MS	36 (97.6%)	17 (98.5%)				
JJ	209 (82.7%)	208 (81.9%)	205 (82.4%)			
GS	0 (99.7%)	52 (96.3%)	36 (97.3%)	215 (82.5%)		
HD	207 (82.7%)	208 (81.9%)	203 (82.5%)	2 (99.7%)	205 (82.7%)	-

(B) ITS1-5.8S-ITS2 of *Oxyrrhis* species.

	O. marina	*O. marina*	*O. marina*	*O. maritima*	*O. marina*	*O. maritima*
	KRR	SH	MS	JJ	GS	HD
KRR	-					
SH	56 (80.9%)					
MS	56 (80.9%)	0 (100%)				
JJ	1 (96.0%)	18 (78.0%)	18 (78.0%)			
GS	0 (100%)	56 (80.9%)	56 (80.9%)	13 (81.9%)		
HD	15 (81.9%)	18 (78.0%)	18 (78.0%)	0 (100%)	68 (82%)	-

(C) LSU of *Oxyrrhis* species.

	O. marina	*O. marina*	*O. marina*	*O. maritima*	*O. marina*	*O. maritima*
	KRR	SH	MS	JJ	GS	HD
KRR	-					
SH	56 (80.9%)					
MS	56 (80.9%)	0 (100%)				
JJ	1 (96.0%)	18 (78.0%)	18 (78.0%)			
GS	0 (100%)	56 (80.9%)	56 (80.9%)	13 (81.9%)		
HD	15 (81.9%)	18 (78.0%)	18 (78.0%)	0 (100%)	68 (82%)	-

2.3.4 Phylogeny

In the phylogenetic trees based on SSU rDNA sequences, the strains of *O. marina* and *O. maritima* formed a clade with AF280077 (UK) strain, and this clade was divergent from a clade including the strain of *O. marina* with AB033717 (JPN), AF482425 (CAN), MS, SH, GS, KRR, and *Oxyrrhis* sp. with WHOI_LH-5-6 (UK) (Fig. 2-5). Furthermore, in the trees based on ITS1, ITS2, 5.8S rDNA sequences, two highly divergent lineages with *O. marina* morphospecies were found. Thus, the JJ strain formed a clade with *O. maritima* HD, whereas it was distant from *O. marina* AB033717 (JPN), MS (Fig. 2-6). When the phylogenetic taxonomic map was drawn using the SSU rDNA and ITS parts, it was found that JJ and HD strains from Jeju region were bound to different groups than the other four species (MS, SH, GS, and KRR). In addition, four strains isolated from the Korean coastal waters showed that a closer relationship with SH, MS, GS, and KRR. The phylogenetic trees showed that *O. marina* belongs to the Lineage I (Clade 1 and 2), while *O. marina* species belongs to the Lineage II (Clade 4). Thus, this present study confirms that these two species are genetically different from each other. Furthermore, I found that the strains of *O. marina* in the Korean coastal waters split into two clades.

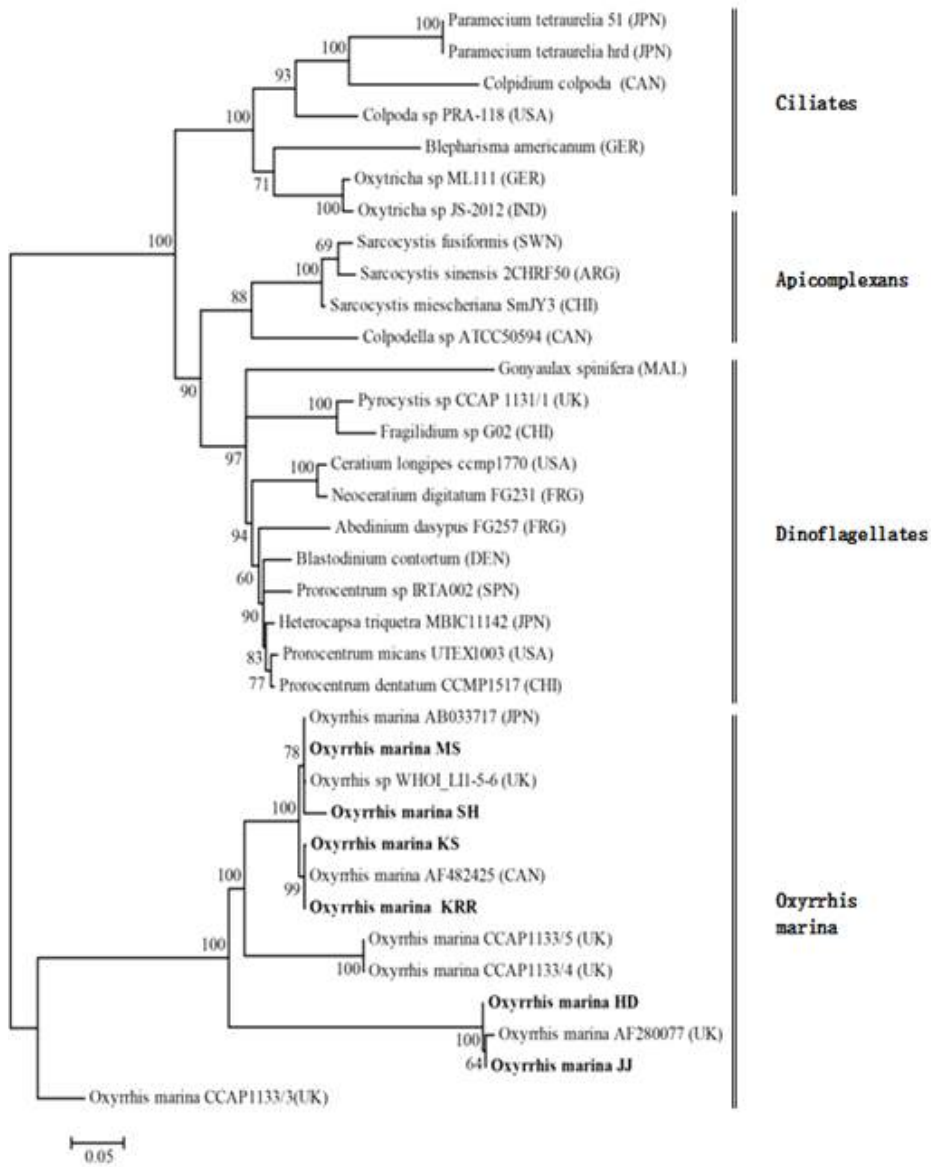

Fig. 2-4. Bayesian tree of SSU ribosomal (r) DNA sequences for 6 strains of *Oxyrrhis* spp. Maximum likelihood bootstrap values are shown above selected nodes. Posterior probabilities ≥ 50 are shown. SSU ribosomal (r) DNA

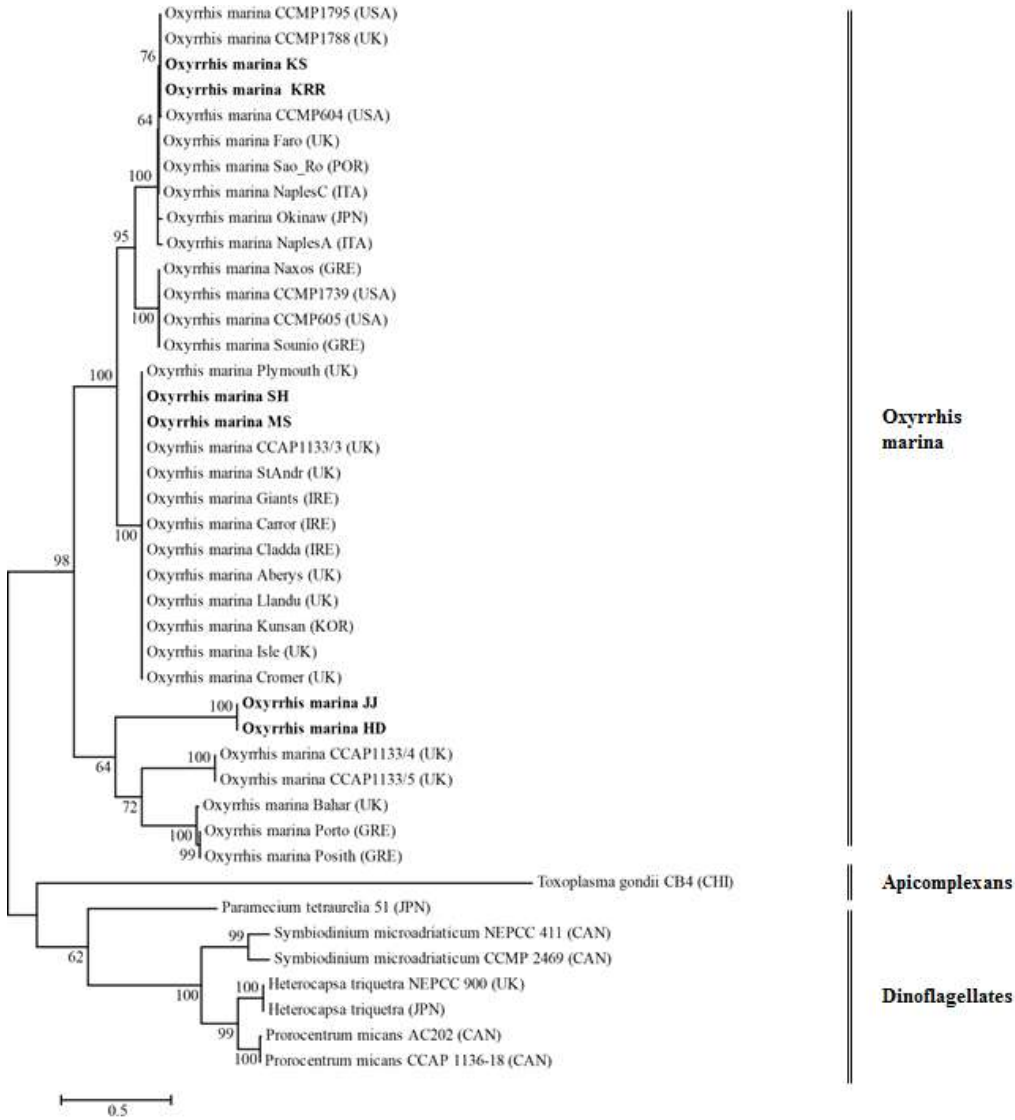

Fig. 2-5. Bayesian tree of internal transcribed spacer 1 (ITS1)-5.8S-ITS2 sequences for 6 strains of *Oxyrrhis* spp. Maximum likelihood bootstrap values are shown above selected nodes. Posterior probabilities ≥ 50 are shown.

2.4. Discussion

2.4.1. Comparison of cell size

In the present study, the length of *O. maritima* was approximately 14% smaller than that of *O. marina*, and the ESD of *O. maritima* was approximately 45% smaller than that of *O. marina*. In the literature, the length of *O. marina* Dujardin (1841) and Kent (1880) were 28 - 51 μm whereas that of *O. maritima* Van Meel (1969) was 16 - 24 μm (Table 2-6). Therefore, the results of this study confirm that the cell size of *O. maritima* is generally smaller than that of *O. marina*.

2.4.2. Phylogeny

Based on ITS1-5.8S-ITS2 phylogenetic trees, *O. marina* from SH and MS species were positioned together with *O. marina* (CCAP 1133/3), i.e., in *O. marina* clade 1, whereas *O. marina* from GS and KRR species were positioned with *O. marina* (CCMP 604), i.e., in *O. marina* clade 2. However, *O. maritima* from JJ and HD strains were present on the same branch as that of *O. marina* (AF280077, UK), which are showed high divergence from *O. marina* (Clade 1 and 2). According to Lowe et al. (2011), SH and MS strains belonged to *O. marina* clade 1, KRR and GS strains belonged to *O. marina* clade 2, and two Jeju strains belonged to *O. maritima* clade 4. Lowe et al. (2010) conducted the phylogenetic analysis of 5.8S rDNA-internal transcribed spacer (5.8S rDNA-ITS) and found two highly divergent lineages within *O. marina* morphospecies, thus proposing the existence of two *Oxyrrhis* species: *O. marina* and *O. maritima*. (Fig. 2-7). In this study, I confirmed that the two *Oxyrrhis* species are genetically

clearly different from each other. Furthermore, I suggest that some strains of *O. marina* (CCAP1133/4, CCAP1133/5, AF280077, Bahar, Porto, GRE) mentioned in previous studies should be transferred to *O. maritima.* It is worthwhile to analyze morphological and genetic characterizations of the strains of *O. marina* and *O. maritima* after collecting them from more diverse environments.

Table 2-6. The designations, and taxonomic authorities, for species in the genus *Oxyrrhis*

Oxyrrhis	**Year**	**Length (μm)**	**Flagella**	**Shape**	**Location**
O. marina Dujardin	1841	44	Several	Oblong, oval bodied, rounded posteriorly	Mediterranean
O. marina Kent	1880	28 – 51	2	Body conical, subcylindrical, rounded posteriorly	St Helier, Jersey
O. marina (starved)	2001	16-31	2	Oblong, oval bodied, rounded posteriorly	Gunsan, Korea
O. phaeocysticola	1900	20	2	Rounded posterior, pointed anterior, excavated oral region with trunk-like projection	Helgoland, Germany
O. tentaculifera Conrad	1939	38	2	More voluminous than O. marina	Belgium
O. maritima Van Meel	1969	16 - 24	2	Twice as long as wide; has a tentacle	Belgium
O. maritima (starved)	2013	15 - 28	2	Body conical, subcylindrical, rounded posteriorly	Jeju, Korea

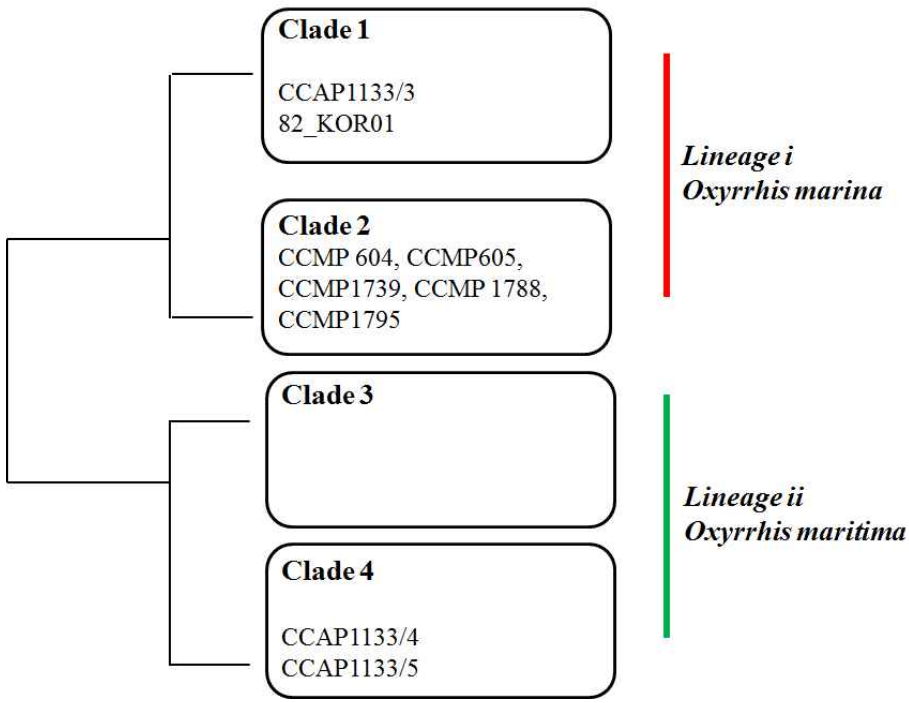

Fig. 2-6. Cladogram (redrawn from Lowe et al., 2010) of the four *Oxyrrhis* clades defined based on 5.8S ITS rDNA and mitochondrial COI sequence data. Representations of the four clades are scaled according to the number of isolates known to belong to each clade. Indicated are the proposed species names for the two *Oxyrrhis* lineages and the most commonly used *Oxyrrhis* strains for which affiliations are known (CCAP and CCMP indicate the source culture collection: CCAP－Culture Collection of Algae and Protozoa, Dunstaffnage, UK; CCMP－Provasoli－Guillard National Center for Culture of Marine Phytoplankton,West Boothbay Harbour, ME, USA)

Chapter 3. Eco-physiology of *Oxyrrhis marina* and *O. maritima* in the coastal waters, salterns, and tide-pools of Korea : effects of temperature, salinity, and light intensity

3.1. Introduction

The eco-physiology of free-living marine heterotrophic protists has been studied intensively on several decades. A single heterotrophic protist species can play diverse roles in marine ecosystems (Weisse and Montagnes 1998; Montagnes and Weisse 2000). The growth rate of each heterotrophic protist is affected by various abiotic factors such as salinity (Droop 1959; Lowe et al., 2005), temperature (Kimmance et al. 2006), and light intensity (Jakobsen et al. 2000). Thus, the physiological study on each species is on eof the critical steps in understanding the structure and function of the ocean ecosystem.

Regarding effects of temperature on the growth of a HTD, (1) the lowest temperature above which the HTD can survive, (2) the highest temperature below which the HTD can survive, and (3) the temperature supporting the maximum growth rate should be determined. In general, with increasing temperature, the growth rates of HTDs increase continuously until the maximum growth rate is achieved, but rapidly decrease at higher temperatures (Montagnes et al. 2003). Furthermore, the lowest and highest temperature for survival or positive growth are generally species dependent (Montagnes et al. 2003). In the temperate regions, temperature largely

fluctuates, which may affect the temporal and spatial distributions of HTDs.

Salinity is also known to affect growth rates of mixotrophic dinoflagellates (MTDs) and HTDs (Band-Schmidt et al. 2004; Maier Brown et al. 2006; Guerrini et al. 2007; Matsubara et al. 2007). Regarding effects of salinity on the growth of a HTD, (1) the lowest salinity above which the HTD can survive, (2) the highest salinity below which the HTD can survive, and (3) the temperature supporting the maximum growth rate should be determined. In general, with increasing salinity, growth rates of HTDs rapidly increase and then become saturated or slowly decrease. The salinity of coastal and estuarine waters fluctuates widely and thus, the growth rates of HTDs may be greatly affected by salinity.

HTDs usually do not need light for their growth if they are provided the enough prey sources. However, *O. marina* (CCMP604) were found to known to express rhodopsins by proteomic analysis in an investigation of potential signal receptors in the cell membrane (Guo et al. 2014). This implies that *O. marina* may have a photo-response mechanism. Regarding light effects on feeding and growth of a HTD, the following questions arise, (1) is the HTD able to grow in darkness when optimal prey is provided? If the growth rate of the HTD on its optimal prey in darkness is similar to that under well-lit conditions, light may not be a critical growth factor. However, if the HTD does not feed or grow well in darkness, but grows well when light is provided, light may stimulate feeding and be a critical growth factor. There have been only a few studies on the growth of MTD or HTD in darkness (Li et al. 2000; Berge et al. 2008); the HTD *Karlodinium armiger* was able to feed on the cryptophyte *Rhodomonas marina* in darkness, but did not grow (Berge et al. 2008).

Because of its easy cultivation and wide distribution in coastal environments, *O. marina* has been used as an 'model species' to examine the responses by heterotrophic dinoflagellates (HTDs) to diverse environmental factors (Droop 1959; Buskey et al. 1998; Johnson 2000; Kimmance 2001). Therefore, there have been many studies on effects of temperature, salinity, and light conditions on the growth rates of *O. marina* (Li et al. 2000; Montagnes et al. 2003; Guerrini et al. 2007). However, there have been no studies on the eco-physiological characterizations of *O. maritima*. Therefore, it is worthwhile to explore this topic. Moreover, difference between the eco-physiological characterizations of these two *Oxyrrhis* species are poorly understood. Thus, I measured and compared the growth rates of *O. marina* and *O. maritima.*

In the present study, we measured the growth rates of *O. marin*a and *O. maritima* at various water temperatures (10-36 ℃), salinities (5-90), and light intensities (0-100 μE $m^{-2} \cdot s^{-1}$). In particular, I tested whether *O. marina* is able to grow in darkness when suitable prey is provided. furthermore, I determined the lowest and highest temperature and salinity at which *O. marin*a or *O. maritima* can survive or grow. The results of the present study provide a basis for understanding the ecophysiologyies of *O. marina* and *O. maritima* and the population dynamics of *O. marina* and *O. maritima* in marine ecosystems.

3.2. Materials and methods

3.2.1 Collection and culture of *Oxyrrhis marina* and *O. maritima.*

Oxyrrhis marina (OMGS_200105) was originally isolated from the coastal waters of Gunsan, while *O. maritima* (OMJJ_201305) from a tide-pool in Jeju Island. Details of the sampling stations were provided in Chapter 2. Cultures of *O. marina* and *O. maritima* were maintained in natural seawaters collected from the coast of Incheon, Korea at a salinity of 31 – 34. Commercialized yeast powder or natural bacterial assemblage was provided as prey every 2-3 days.

Clonal cultures of *O. marina* (Gunsan) and *O. maritima* (Jeju) were established by two serial single cell isolations. The phototrophic dinoflagellate *Amphidinium carterae* (5,000-8,000 cells mL^{-1}) or dried yeast was provided as prey. As the concentration of *O. marina* and *O. maritima* increased, the grazers were subsequently transferred to 32-, 270-, and 500-mL polycarbonate (PC) bottles containing dried yeasts. The bottles were again filled to capacity with freshly filtered seawater, capped, and placed on the shelf at 20 ℃ under 20 μE m^{-2} s^{-1} of continuous cool white fluorescent light. Once dense cultures of *O. marina* and *O. maritima* were obtained, they were transferred to 500-mL PC bottles with dried yeast as prey every 7d. In this experiment, to create identical prey conditions for the temperature, salinity, and light intensity experiments, I provided autoclaved dried yeasts instead of the algal prey.

3.2.2. Temperature effects (tolerance and optimal temperature)

Experiment 1 and 2 were designed to investigate effects of temperature on the growth rates of *O. marina* (Gunsan) and *O. maritima* (Jeju) fed autoclaved dried yeast (ca. 20 mg per liter) at a single prey concentration. Temperatures of 5, 10, 15, 20, 25, 30, 35, and 36 ℃ were established to determine the lowest and highest temperatures at which *O. marina* or *O. maritima* can survive and the temperature supporting the maximum growth rate should be determined.

Each of 5 one-liter PC bottle containing *O. marina* or *O. maritima* was placed on a shelf in one of five culture rooms (1m × 1.5m × 1.2m) maintained at the target temperatures and of 20 μE m^{-2} s^{-1} of continuous cool white fluorescent continuous light. The bottles were taken from the culture rooms and then dried yeasts were provided as prey every 1-2 d. The bottles were placed back on the shelf. *O. marina* or *O. maritima* cells were acclimated to the target temperatures except < 5 and > 35 ℃ for more than 10 days. For the target temperatures < 5 ℃, *O. marina* or *O. maritima* cells acclimated at 10 ℃ for 10 d were used without further acclimation. Similarly, For the target temperatures > 35 ℃, *O. marina* or *O. maritima* cells acclimated at 30 ℃ for 10 d were used without further acclimation. In this pre-incubation period, one-mL aliquots were taken from each bottle everyday and then the abundance of *O. marina* or *O. maritima* were determined using a compound microscope.

In experiment 1 and 2, triplicate 270-mL culture flasks (SPL, containing mixtures of predator and prey), triplicate prey control bottles (containing prey only), and triplicate predator control bottles (containing predators only) were set up for each target temperature. The initial

abundances of *O. marina* and *O. maritima* (ca. 200 cell mL^{-1} at temperature of 15, 20, 25, and 30 ℃; 700 cell mL^{-1} at 10, 32, 35 and 36℃) and the concentrations of autoclaved dried yeasts (ca. 20 mg per liter) were established using an autopipette to deliver predetermined volumes of known cell concentrations to the bottles.

To determine the actual initial predator densities (cells mL^{-1}) at the beginning of the experiment and after a 4 d incubation, 5 ml aliquots were removed from each bottle and fixed with 5% Lugol's solution, and all *O. marina* or *O. maritima* cells and all or > 300 prey cells in the three 1 mL SRCs were enumerated. Prior to taking subsamples, the conditions of *O. marina* or *O. maritima,* and prey were assessed under a dissecting microscope.

The specific growth rate of *O. marina* or *O. maritima*, μ (d^{-1}), was calculated as follows:

$$\mu = \frac{\ln(H_t / H_0)}{t}$$

where H_o is the initial concentrations (cells ml^{-1}) of *O. marina* or *O. maritima*, and H_t is the final concentration after time t (d^{-1}).

3.2.3. Salinity effects (tolerance and optimal salinity range)

Experiments 3 and 4 were designed to investigate the effects of salinity on the growth rates of *O. marina* and *O. maritima.* For the experiments, artificial seawater (S 9883 Sea salts, Sigma-Aldrich, St. Louis,

USA) or sterilized distilled water were added into sterilized seawater to obtain final salinities of 5, 10, 20, 33, 50, 70, and 90. Each bottles was placed on the shelf in a culture room maintained at 20 ℃.

The bottles were taken from the culture rooms and then dried yeasts were provided as prey every 1-2 d. the bottles were placed back on the shelf. *O. marina* or *O. maritima* cells were acclimated to the target salinities except < 5 and > 70 for more than 10 days. For the target salinities < 5, *O. marina* or *O. maritima* cells acclimated at the salinity of 10 for 10 d were used without further acclimation. Similarly, for the target salinity > 70, *O. marina* or *O. maritima* cells acclimated at the salinity of 70 for 10 d were used without further acclimation. In this pre-incubation period, one-mL aliquots were taken from each bottle everyday and then the abundance of *O. marina* or *O. maritima* were determined using a compound microscope.

Triplicate 270 mL culture flasks (SPL, containing mixtures of predator and prey), triplicate prey control bottles (containing prey only), and triplicate predator control bottles (containing predators only) were set up for each target salinity. The initial abundances of *O. marina* and *O. maritima* (ca. 300 cell mL^{-1} at salinities of 10, 20, 33 and 50; 500 cell mL^{-1} at salinities of 10, 70 and 90) and the concentrations of autoclaved dried yeasts (ca. 20 mg dried yeast per liter) were established using an autopipette to deliver predetermined volumes of known cell concentrations to the bottles. The actual initial abundances of *O. marina* and *O. maritima* (cells mL-1) at the beginning of the experiment and after a 4 d incubation were determined described above. Furthermore, the specific growth rate of *O. marina* or *O. maritima* at each target salinity was also calculated described above.

3.2.4. Light effects

Experiments 5 and 6 were conducted to investigate the effects of light intensity on the growth rates of *O. marina* and *O. maritima* when fed dried yeast (ca. 4mg dried yeast per 200 mL) as prey. Dense cultures of *O. marina* and *O. maritima* were grown on dried yeast at 10 μE $m^{-2}s^{-1}$ of continuous cool white fluorescent light, without any mixing. Bottles were wrapped with an aluminum foil for darkness and place on the shelf for each target light intensity to provide 0, 1, 20, or 100 μE $m^{-2}s^{-1}$, respectively. The experimental bottles were maintained at 20 ℃, and every 1 - 2 d, the contents of each bottle were evenly distributed into two 1-L PC bottles containing the target amount of dried yeast, and placed back on the shelf. In this manner, *O. marina* and *O. maritima* cells were acclimated to their target light intensities for 10 d. Initial abundances of *O. marina* and *O. maritima* (ca. 200 cell mL^{-1} at light intensities of 0, 1, 20 and 100 μE $m^{-2}s^{-1}$) and autoclaved dried yeast were established using an autopipette to deliver a predetermined volume of culture with a known cell density to the experimental bottles. Triplicate 270 mL culture flasks (SPL, containing mixtures of predator and prey), triplicate prey control bottles (containing prey only), and triplicate predator control bottles (containing predators only) were set up for each salinity.

The actual initial abundances of *O. marina* and *O. maritima* (cells mL^{-1}) at the beginning of the experiment and after a 4 d incubation were determined described above. Furthermore, the specific growth rate of *O. marina* or *O. maritima* at each target light intensity was also calculated described above.

3.3. Results and Discussion

3.3.1. Temperature effects.

O. marina did not grow at the temperatures ≥ 35℃ (Fig. 3-1). With increasing temperature from 10 to 25 ℃, the growth rate of *O. marina* increased continuously from 0.26 d^{-1} at 10 ℃ to 0.96 d^{-1} at 25 ℃, but it rapidly decreased between 30 and 35 ℃ (Fig. 3-1). However, The strains of *O. maritima* grew at the temperatures of > 35 ℃, but *O. marina* did not grow. Furthermore, the optimal temperature supporting the maximum growth rate of *O. marina* was 25 ℃, while that of *O. maritima* was 30 ℃. With increasing temperature from 10 to 30 ℃, the growth rate of *O. maritima* increased continuously from 0.23 d^{-1} at 10 ℃ to 1.11 d^{-1} at 30 ℃, but it rapidly decreased between 32 and 36 ℃. The growth rates of both *O. marina* and *O. maritima* were significantly affected by temperature (ANOVA test, $p<0.05$, Fig. 3-2). At the temperature 35 ℃ the growth rates of *O. maritima* was significantly greater than those of *O. marina* ($p < 0.05$, one tailed t-test). However, the temperature of 10, 15, 20, 25, 30, and 32 ℃, the growth rates of *O. marina* were not significantly different from those of *O. maritima* ($p > 0.05$, two-tailed t-test).

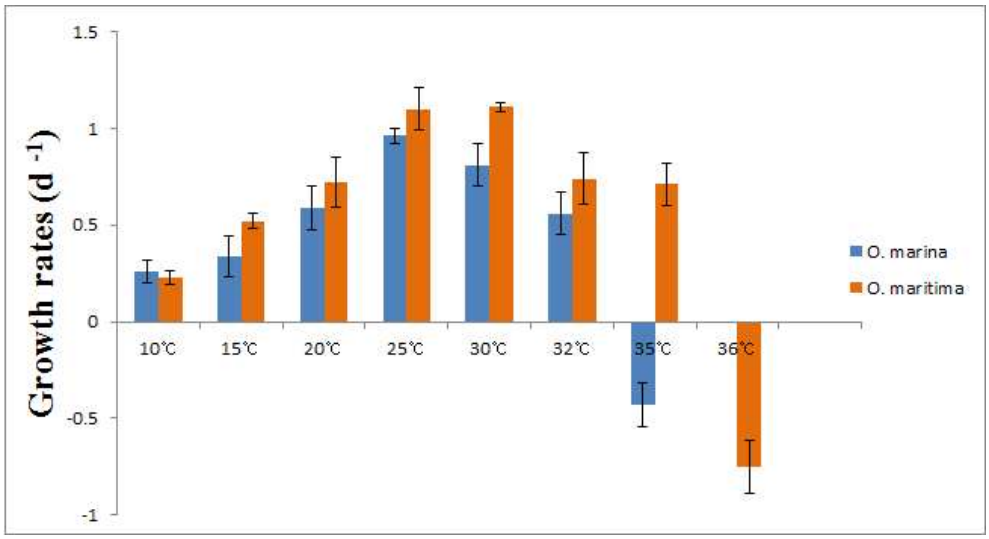

Fig. 3-1. The growth rates (d^{-1}) of *O. marina* and *O. maritima* under the various temperatures. The error bar represents the standard deviation from the mean of triplicate data (n = 3). The p values in all were $p<0.05$ (ANOVA test).

3.3.2. Salinity effects

O. marina did not grow at the salinities of < 4. With increasing salinity from 5 - 50, the growth rate of *O. marina* increased from 0.37 d^{-1} at 5 to 0.67 d^{-1} at 50, but it rapidly decreased between 50 and 90 (Fig. 3-2). However, *O. maritima* species could grow at the salinity of 2. The growth rates of both *O. marina* and *O. maritima* were significantly affected by salinity (ANOVA test, $p<0.05$, Fig. 3-2). At the salinities of 5, 10, and 20, the growth rates of *O. maritima* were significantly greater than those of *O. marina* ($p < 0.05$, one tailed t-test), while at the salinities of 90 the growth rates of *O. maritima* was significantly lower than those of *O. marina* ($p < 0.05$, one tailed t-test). However, the salinities of 33, 50, 70,

the growth rates of *O. marina* were not significantly different from those of *O. maritima* ($p > 0.05$, two-tailed t-test).

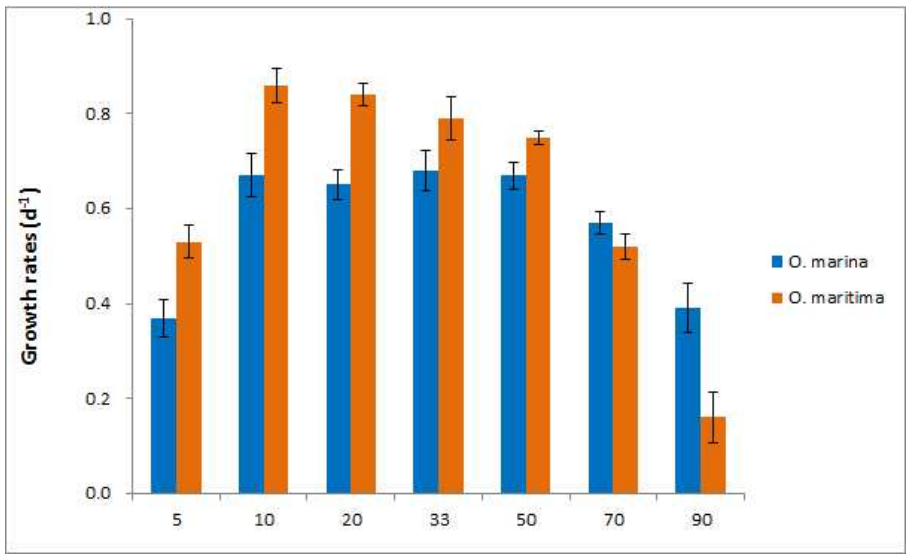

Fig. 3-2. The specific growth rate (d^{-1}) of *O. marina* and *O. maritima* under various salinities The error bar represents the standard deviation from the mean of triplicate data (n = 3). The p values in all were $p<0.05$ (ANOVA test).

3.3.3. Light intensity.

At the light intensities of 0-100 $\mu Em^{-2}s^{-1}$, the growth rates of *O. marina* fed dried yeasts did not largely change (i.e., 0.58 d^{-1} at 0 $\mu Em^{-2}s^{-1}$ to 0.59 d^{-1} at 100 $\mu Em^{-2}s^{-1}$). However, with increasing light intensity from 0 to 100 $\mu Em^{-2}s^{-1}$, the growth rate of *O. maritima* decreased from 0.85 d^{-1} at 0 $\mu Em^{-2}s^{-1}$ to 0.59 d^{-1} at 100 $\mu Em^{-2}s^{-1}$ (Fig. 3-3). The growth rates of both *O. marina* and *O. maritima* were not affected by light intensity (ANOVA test, $p > 0.1$, Fig. 3-3). In addition, the growth rate of *O. marina* was not significantly different than that *O. maritima* ($p > 0.1$, two-tailed t-test).

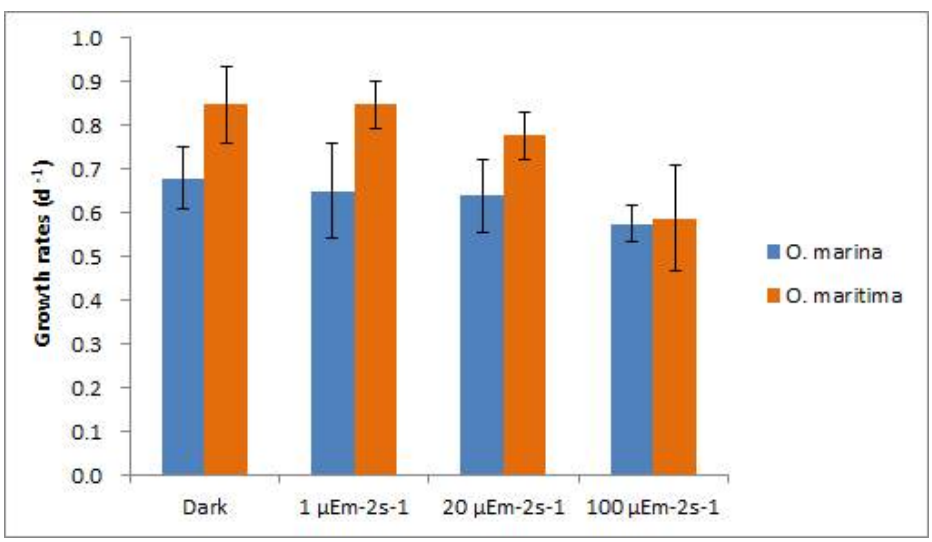

Fig. 3-3. The specific growth rate (d^{-1}) of *O. marina* and *O. maritima* under various light intensities. The error bar represents the standard deviation from the mean of triplicate data (n = 3). The p values in all were $p > 0.1$ (ANOVA test) and $p > 0.1$ (t-test, two-tailed t-test)

O. marina did not grow at 5 ℃ and ≥ 35 ℃, while *O. maritima* is able to grow at 5 ℃ and 35 ℃. Therefore, the temperature range of positive growth for *O. maritima* was wider than for *O. marina.* Water temperatures in temperate areas including Korea usually decrease to 5 ℃ in winter and increase to > 35 ℃ in summer. Therefore, in temperate waters, water temperature could be a critical factor differently affecting the abundances of *O. marina* and *O. maritima*. The lower temperature limit (LTL) and upper temperature limit (UTL) for the positive growth of *O. marina* and *O. maritima* were higher than some other HTDs such as C*ryptoperidiniopsis brodyi, Pfiesteria piscicida,* and *Luciella masanensis* (Baek et al. 2010). *O. maritima* were isolated from tide pool where the is more cooling and heating than in general harbors.

Chapter 4. Abundance and distribution of *Oxyrrhis marina* and *O. maritima* in Korean waters using real time PCR(qPCR) and digital PCR (dPCR)

4.1. Introduction

For a long time, estimating the biomass of nano-, micro-, and zooplankton have been based traditionally on cell counts and microscopic measurements of cell size. Lugol's iodine (Throndsen, 1978) is a widely used fixative and is recommended commonly for preserving ciliates and cflagellates (Throndsen, 1978; Leakey et al., 1994; Karayanni et al., 2004; Zarauz et al., 2008). However, it is been limiting to accurate analysis because there are many species with similar morphologies under microscopy, such as *Cochlodinium polykrikoides, Gyrodinium impudicum,* and *Gymnodinium catenatum* (Stoecker et al., 1994; Lee et al., 2001). However, direct counting based on morphological features, requires labor, time, and significant skill. In addition, the morphology of the algae might change depending on the environmental conditions or their growth phase (Kamikawa et al., 2006).

There are a variety of plankton species in the ocean, some of which can cause problems to human life. For example, red tides or harmful algal blooms (HABs) can cause large-scale fish mortality, and can consequently have substantial economic effects on the aquaculture industry (Park et al. 2013; Lee et al., 2017). To reduce these problems, understanding the marine ecosystem based on species-specific relationships is important. Therefore,

there have been many studies on quantifying the abundance of target-species in natural seawater.

For the past several decades, the genetic analysis method has been in development, under the consideration that genetic information is different even if species are morphologically similar. This is because, unlike algal morphology, the sequences of genes do not change depending on the environmental conditions or algal growth phase. Hence, many studies have tried to apply a rapid, accurate, and simple detection and quantification method by using a real-time PCR assay with a TaqMan probe/or SYBR Green dye to monitor the target species. (Kamikawa et al., 2006). The technique was developed by real-time PCR from general PCR. Over the past decade, real-time PCR (qPCR) has been widely used for the identification, detection, and quantification of abundances of marine organisms such as bacteria, protists, and algae. This technique provides highly sensitive, quantitative, and rapid detection of target organisms (Park and Park, 2010).

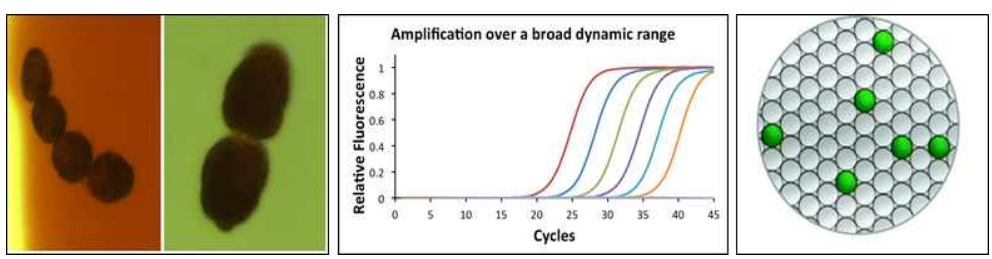

Fig. 4-1. Methods for quantification of target-species. (A) Direct counting, (B) real-time PCR (qPCR), and (C) digital PCR (dPCR).

In recent years, real-time PCR has improved to digital PCR (dPCR). This third generation of PCR has gained recognition in biomedical research as it provides a platform for the precise and accurate quantification of nucleic acids without the need for a standard curve. Moreover, disclosed is a method for accurately determining the number of template molecules in a library of nucleic acids (e.g., DNA) to be sequenced (Fig. 4-2). The method does not require large amounts of the DNA sample, nor does it require the preparation of a standard curve. The method is especially applicable to methodologies for "sequencing by synthesis," where quantification of the starting library is important. The method uses quantitative real time PCR, especially digital PCR, which measures the number of individual molecules in a sample. The present method particularly may use a microfluidic device for running large numbers of PCR reactions. Each PCR reaction is monitored in real time by a primer/probe combination. The forward primer is adapted to contain a sequence not on the adapter but which corresponds to a probe sequence. A short probe which generates fluorescence during the PCR process is used

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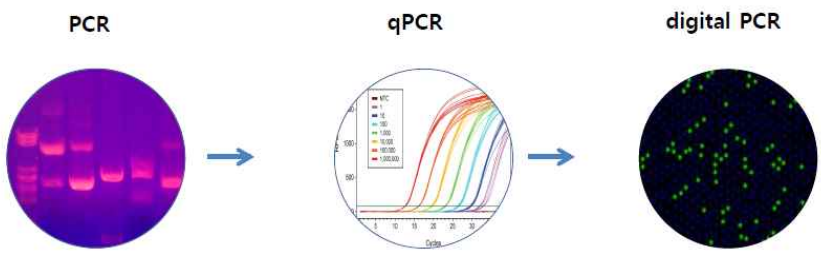

Fig 4-2. PCR technologies (semi-quantification-relative quantification -absolute quantification)

By improving the genetic technology, digital PCR (dPCR) was developed from real time PCR. qPCR (second generation PCR) is relative quantification, while the dPCR (third generation PCR) is absolute quantification without standard gene (Fig. 4-3). I applied the qPCR and dPCR assay to environment samples.

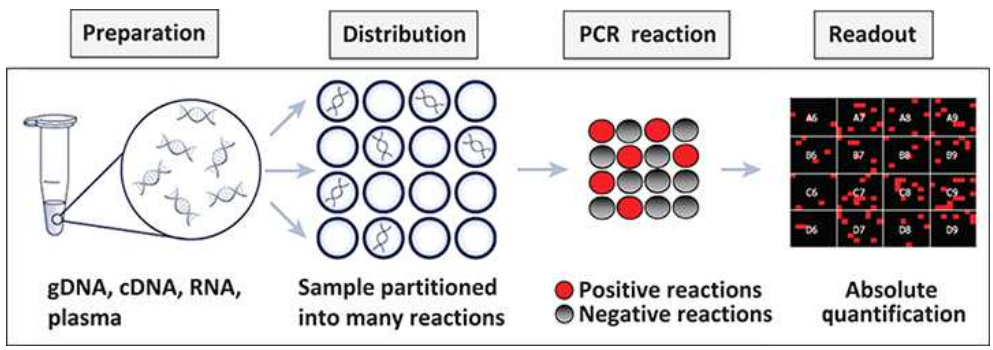

Fig. 4-3. A methods for the analysis the digital PCR (dPCR)

4.2 Materials and methods

4.2.1. Sampling stations

To explore the distribution of *O. marina* and *O. maritima* in Korean waters, water sample were collected at seven harbors on the west coast (Shiwha, Dangjin, Taean, Seocheon, Gunsan, Buan, and Mokpo), 10 harbors on the south coast (Jangheung, Koheung, Yeosu, Kwangyang, Tongyoung, Masan, Jinhae, Geoje, Busan, and Gijang), six harbors on east the coast (Ulsan, Pohang, Uljin, Samcheok, Donghae, Joomoonjin, and Sokcho), and six harbors on Jeju Island (Aewol, Hanlim, Seogwipo, Pyoseon, and Seongsan) in January, March, May, July, August, October, and December, 2016.

Furthermore, I also take environmental samples from tide-pool and Saltern in Taean and Jeju Island on June and September in 2016. The water temperature and salinity were broader than water samples from general harbors. Using sampling bucket, water samples were collected from the surface and/or bottom waters at each station.

4.2.2. Extraction for field sample for real-time PCR and digital PCR

The field samples for analysis their distributions and abundance were extracted with AccuPrep® Genomic DNA Extraction Kit by adjusting the proteinase K and binding buffer (GC) reagents volume (Bioneer Cooperation, Daejeon, Korea) as follows : Add 40 µl of Proteinase K to tubes containing field sample and add 400 µl of Binding buffer (GC) to the sample and mix

immediately by vortex mixer. Incubate at 60℃ for 10 min and add 100 µl of Isopropanol and mix well by pippetting. After this step, briefly spin down to get the drops clinging under the lid. Carefully transfer the lysate into the upper reservoir of the Binding column tube (fit in a 2 mL tube) without wetting the rim. Close the tube and centrifuge at 8,000 rpm for 1 min. Open the tube and transfer the Binding column tube to a new 2 mL tube for supplied filtration. Add 500 µl of Washing buffer 1 (W1) without wetting the rim, close the tube, and centrifuge at 8,000 rpm for 1 min. Open the tube and pour the solution from the 2 mL tube into a disposal bottle. Carefully add 500 µl of Washing buffer 2 (W2) without wetting the rim, close the tube, and centrifuge at 8,000 rpm for 1 min. Centrifuge once more at ca. 12,000 rpm for 1 min to completely remove ethanol, and check that there is no droplet clinging to the bottom of Binding column tube. Residual W2 in the Binding column tube may cause problems in later applications. Transfer the Binding column tube to a new supplied 1.5 mL tube for elution, add 200 µl of Elution buffer (EL, or nuclease-free water) onto Binding column tube, and wait for at least 1 min at room temperature (15~25℃) until EL is completely absorbed into the glass fiber of Binding column tube. To increase DNA yield, you should wait for 5 min after adding Elution buffer (EL). The volume of EL added 100 µl.

4.2.3. Design of Dual-Labeled BHQplus Probes and Primer Sets for qPCR

Ribosomal DNA sequences (SSU, ITS1-5.8S-ITS2, and LSU) were used to construct species-specific primers and probes to detect Oxyrrhis marina and O. maritima from the environmental population. These were subsequently compared to published sequences using a BLAST homology search based on GenBank. The primers and probes were dual-labeled with

the fluorescent dyes FAM and BHQplus (Biosearch Technologies Inc., Novato, CA, USA) at the 5′ and 3′ ends. The specificity of each of the Oxyrrhis species-specific primers and probe sets were also tested using concentrated rDNA extracted from 10,000 cells of each species. The DNA extraction method used on these cultured isolates was the same as the one used for field-collected samples (see above).

4.2.4. Real-time PCR assay conditions and standard curve

Samples for standard curves of cell number equivalents were generated using each target concentration of DNA extracted from a known number of cells collected from *O. marina* and *O. maritima* cultures. qPCR reactions were performed using 1 μL of template DNA combined with 0.2 μM forward and reverse primers, 0.15 μM probe, 5 μL of HiFast Probe Hi-Rox (Genpole, Gwangmyung, Korea), and PCR-grade water (total final volume = 10 μL). The thermal cycle detection was accomplished using 50 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by melt curve analysis and then a hold at 4 °C (Eco 48 Real Time PCR system, Germany, Fig. 4-2).

Standard curves for TaqMan-based qPCR assays were obtained using the DNA extracted from a known cell number for each species. The cell density of *O. marina* and *O. maritima* were calculated by comparing Ct values and the qPCR standard curves determined for each species.

Fig. 4-4. Eco 48 Real Time PCR system

4.2.5. Performing the digital PCR (dPCR)

I compared the gene size of O. marina and O. maritima by using the Clarity™ digital PCR system (JN Medsys, Singapore). The accuracy and precision of the Clarity™ dPCR system were assessed by quantifying the single-copy gene from dilutions of genomic DNA with expected target concentrations ranging from 0 – 33,600 copies/15 µL of reaction mixture.

Each reaction mix consisted of 0.25 µM forward and reverse primers, 0.25 µM probe, 1× FastStart Essential DNA Probes Master (Roche), 1× Clarity™ JN solution, and 3 µL of DNA sample, topped up to 15 µL with PCR grade water. The JN solution is a proprietary formulation optimized for robust performance on Clarity™ high-density chips. Using the Clarity™ auto loader, the resultant mix was then delivered onto the chip, where it was sub-divided into 10,000 partitions. The partitions were then sealed with the ClarityTM Sealing Enhancer and 230 µL of Clarity™ Sealing Fluid, followed by thermal cycling using the following parameters: initial cycle of 95 °C for

5 min and 40 cycles of both 95 °C for 50 s and 58 °C for 90 s (ramp rate = 1 °C/s). After PCR amplification, the tube strips were transferred to the Clarity™ Reader, which detects fluorescent signals from each partition concurrently. The data were analyzed with the Clarity™ software (version 1.0), and a proprietary algorithm was used for setting each threshold based on the fluorescent intensities to determine the proportion of positive partitions out of the total. Based on this information, the software determines the DNA copies per microliter of dPCR mix, using Poisson distribution. The mean partition volume of 1.336 nL was used for copy number calculation (Low et al. 2017).

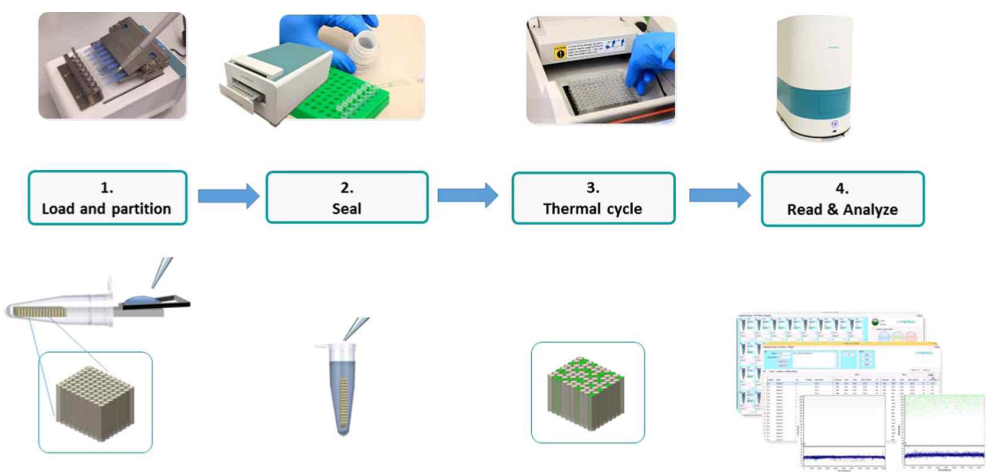

Fig 4-5. Clarity™ digital PCR system (Bio-D company).

4.2.6. Data analysis

After PCR amplification, the tube strips were transferred to the Clarity™ Reader, which detects fluorescent signals from each partition concurrently. The data were analysed with the Clarity™ software (version 1.0), and a proprietary algorithm was used to set each threshold based on fluorescent intensities in order to determine the proportion of positive partitions out of the total. Based on this information, the software determined the DNA copies per microlitre of dPCR mix using Poisson statistics. The mean partition volume of 1.336 nL was used for copy number calculation (Low et al. 2017).

4.3. Results and Discussion

4.3.1. real-time PCR (qPCR)

Species-specific primers and probe for the analysis the qPCR and dPCR of *O. marina* and *O. maritima* were designed (Table 4-1). Sequences of *O. marina* and *O. maritima* species-specific primers and probes were used for dPCR analysis in this study.

Table 4-1. Sequences of *Oxyrrhis marina* and *O. maritima* species-specific primers and probes used for dPCR analysis in this study.

Species	Primer/Probe	Sequences (5′->3′)	Probe
Oxyrrhis marina	Forward	GGAGCGGCAGGATCTACAC	
	Reverse	GCTCGCTGCATTGCTAGACT	
	Probe	TCACGGTGCCAGCAGT	FAM
Oxyrrhis maritima	Forward	TGATGCTAGGACCCGAAAG ATG	
	Reverse	TGCACGTCAGTATCGCTACA AG	
	Probe	AACTATGCTTGGGTAGGGTG AAA	FAM

To analyse the cell abundance in the environment, the standard curves were used for *O. marina* (A, B) and *O. maritima* (C, D) (Fig. 4-6).

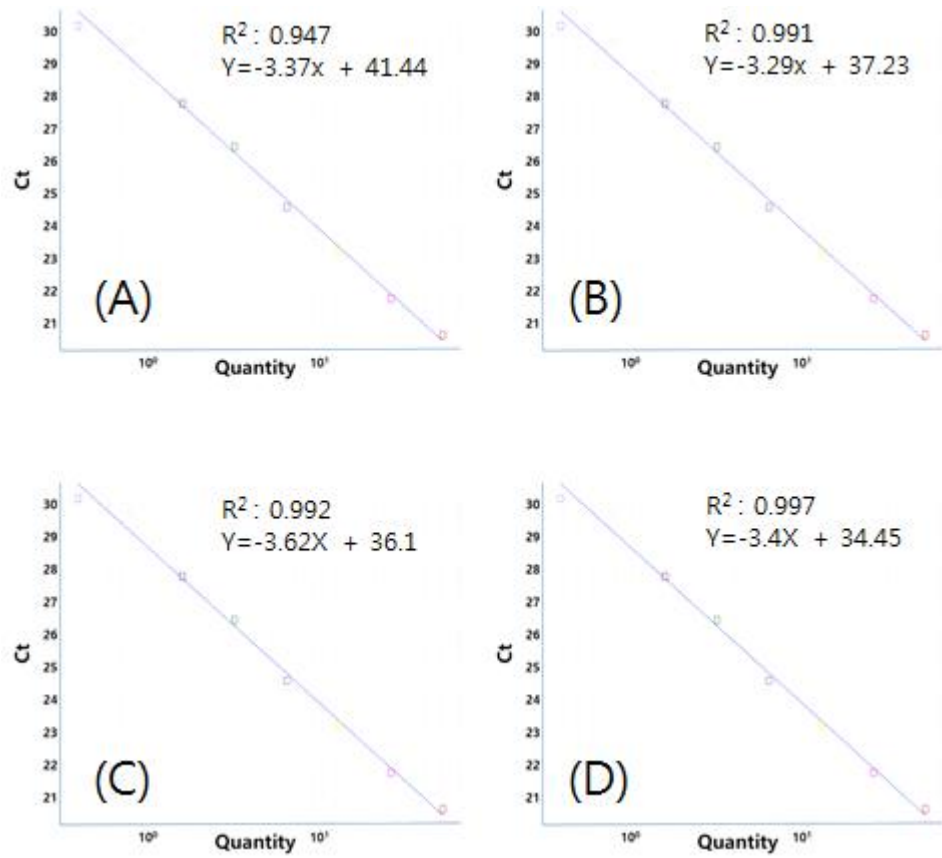

Fig. 4-6. Determination of real-time PCR efficiencies of the target gene (ITS). Ct cycles versus gDNA concentration input were plotted to calculate the slope (mean ± SD; n = 2).

4.3.2. Digital PCR (dPCR)

In the present study, we quantified the *O. marina* and *O. maritima* based on dPCR with species-specific primer and probe. To determine the copy number of target DNA (gDNA) per species, laboratory culture was used. Copy number concentrations measured by Clarity™ correlate well with measurements determined by the BioMark™ HD and QX100™ dPCR systems. Total copy number of *O. marina* and *O. maritima* were highly linearly correlated with cell number (r^2=0.996 and 0.997), and no significant difference in the average number of copies per cell was detected the between cell numbers. A single cell of *O. marina* produced an average of 138 - 158 (147 ± 6.9) copies of the target DNA fragments (Fig 4-7; Table 4-2). In the case of *O. maritima* an average of 167 - 193 (177 ± 9.2) copies per cell were produced (Fig 4-7; Table 4-2). The ESD of *O. maritima* is smaller than *O. marina.* However, there was no positive correlation between the copy numbers per species and cell size in both *O. marina* and *O. maritima*

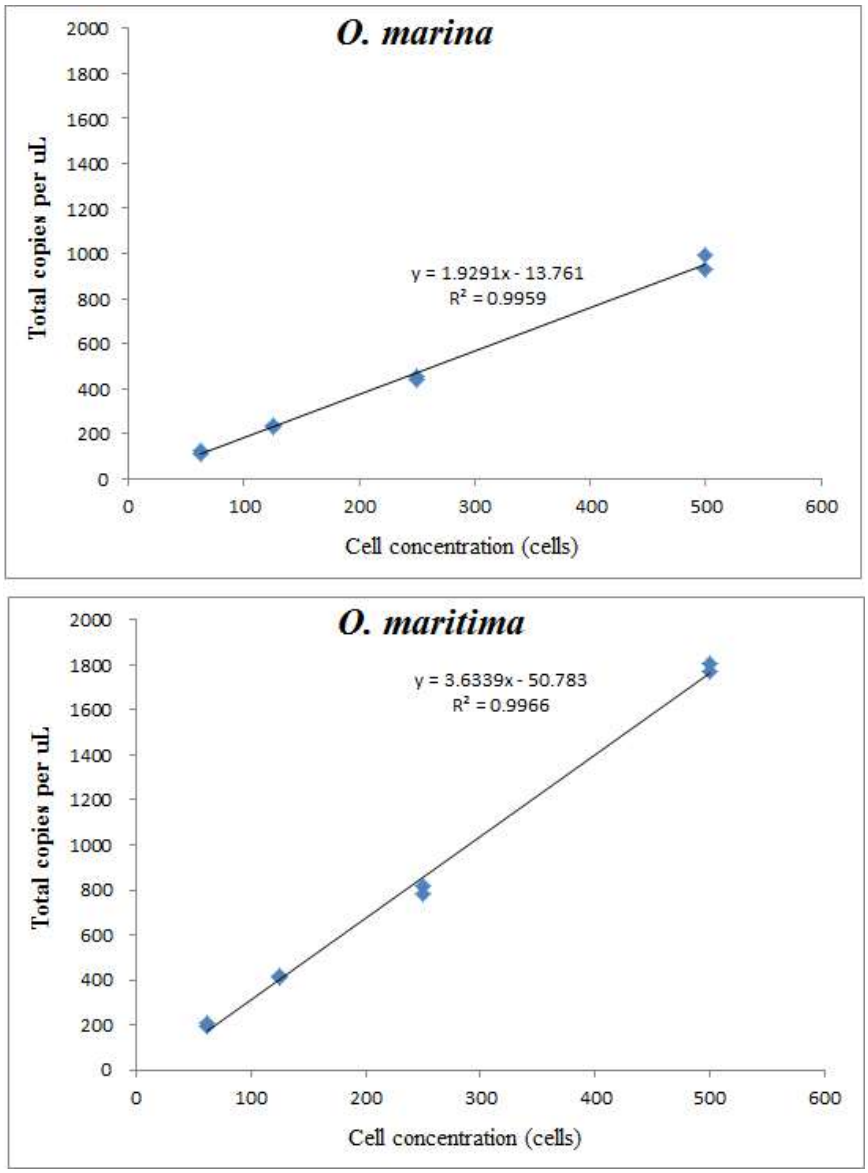

Fig. 4-7. Standard curves from digital PCR (dPCR) of the target genes (ITS) were used for the determination of number of copies per cell of *O. marina* and *O. maritima.* Cell concentration (cells) versus total copies per μL were plotted to calculate the slope

Table 4-2. Calculation of the specific copy number per *Oxyrrhis marina* (A) and *O. maritima* (B) cells. Digital PCR was performed on culture samples containing various known cell numbers.

(A) *Oxyrrhis marina*

cell counting (cell)	total copies uL^{-1}	copies $cell^{-1}$
62.5 -1	108	138
62.5 -2	122	156
125 -1	233	149
125 -2	232	148
250 -1	455	140
250 -2	438	146
500 -1	929	149
500 -2	990	158
Average		**148 ± 6.9**

(B) *Oxyrrhis maritima*

cell counting (cell)	total copies uL^{-1}	copies $cell^{-1}$
62.5 -1	206	176
62.5 -2	196	167
125 -1	409	175
125 -2	416	178
250 -1	783	167
250 -2	817	174
500 -1	1804	192
500 -2	1774	189
Average		**177 ± 9.2**

4.3.3. Environmental samples

The temporal variations in abundance of *O. marina* and *O. maritima* in Korean coastal waters was measured using real-time PCR (qPCR). The abundance of the species was higher in March, May, and July than in January and December when the water temperature was low. In addition, the abundance of *O. maritima* was much higher than *O. marina* throughout the year. During this study period at 29 coastal stations in Korea, *O. marina* was present only four times in December on the southern coasts and near the Jeju Island in Gerje (GJ), Tongyoung (TY), Aewol (AW), and Seogwipo (SW) (Fig. 4-8). *O. maritima* was present from January to December when water temperature was 8.7−24.9 ℃. The maximum abundance of *O. maritima* was 38 cells ml^{-1} in Busan Bay. This value is higher than the maximum abundance of *O. marina*, which was 3 cells ml^{-1} in Tongyoung bay (Fig. 4-9). These environmental data supported the laboratory experiment, which indicated that *O. maritima* have more effective growth rates under extreme temperature conditions.

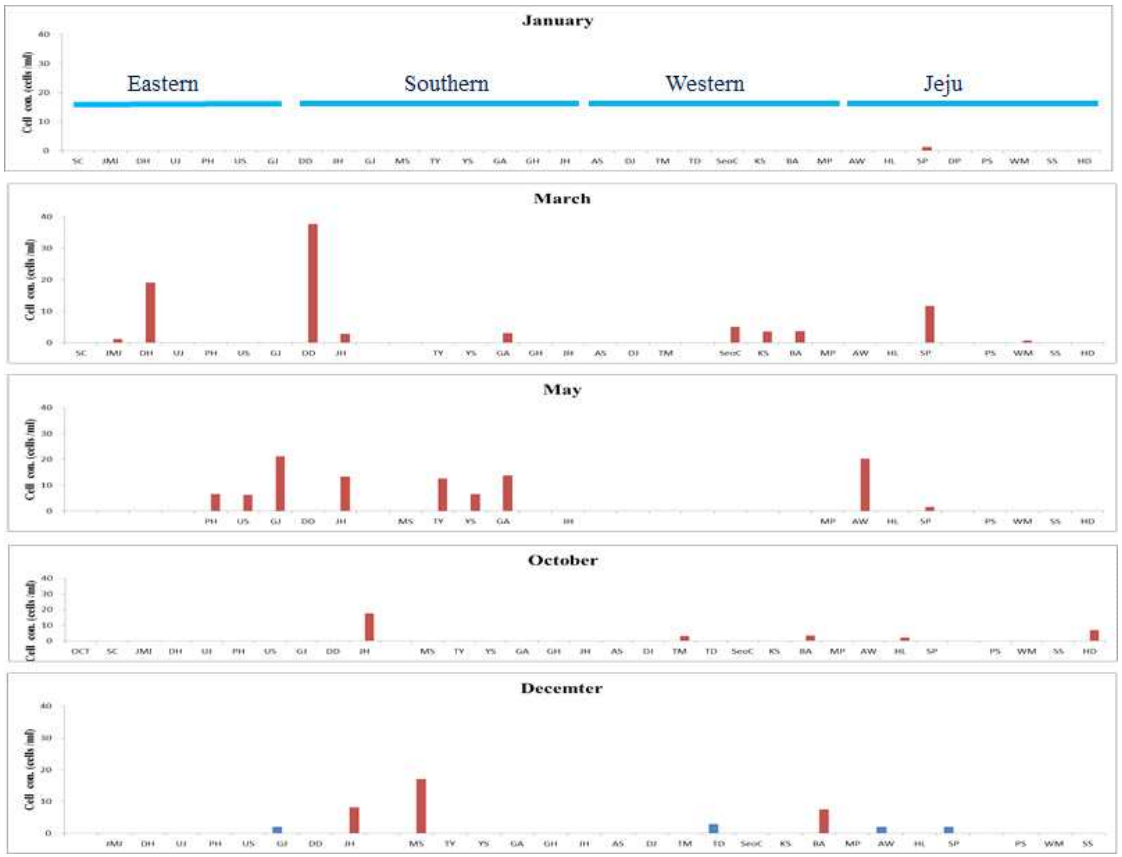

Fig. 4-8. The temporal variations in the abundances of *O. marina* and *O. maritima* from 29 stations in Korean coastal waters in January, March, May, October, and December, 2016

The temporal variations in abundance of *O. marina* and *O. maritima* in tide pools and salterns were measured using qPCR and dPCR. Most of the environments (except only > 30−50), the distribution of *O. maritima* was much wider than *O. marina.* During this study period, the maximum abundance of *O. marina* was 7491 cells ml^{-1} in Jeju tide pool. This value is higher than the maximum abundance of *O. maritima*, which was 3520 cells ml^{-1} in Jeju tide pool (Fig. 4-9; 4-10). *O. maritima* and *O. maritima* were present from June and September (this study period) when water salinity was 9-101 and 26-75, respectively. These environmental data supported the laboratory experiment, which indicated that *O. maritima* have more effective growth rates under extreme salinity conditions.

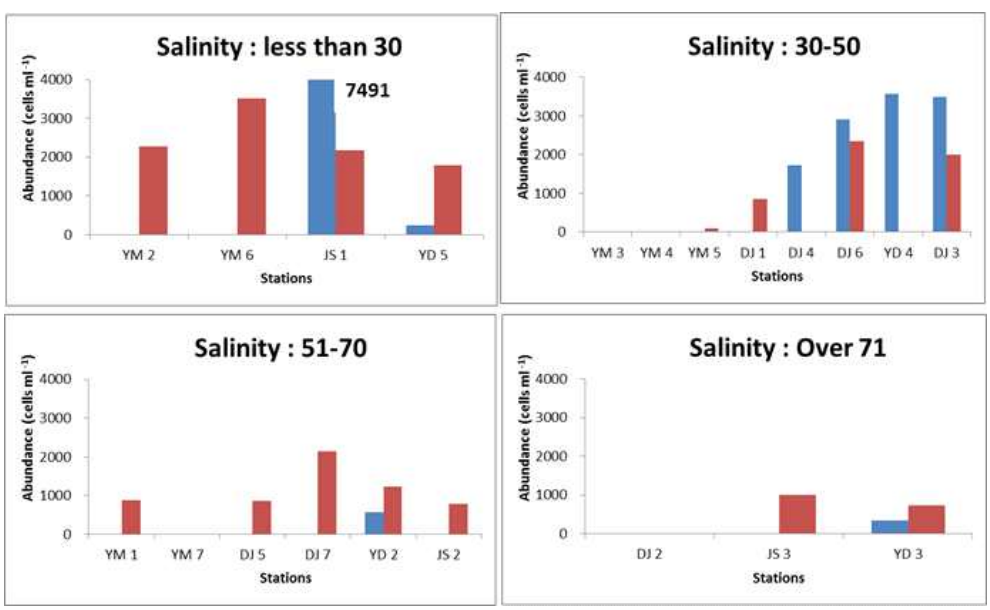

Fig. 4-9. The abundances of *O. marina* and *O. maritima* under different salinity ranges. (A) < 30, (B) > 30−50, (C) > 51−70, and (D) > 70.

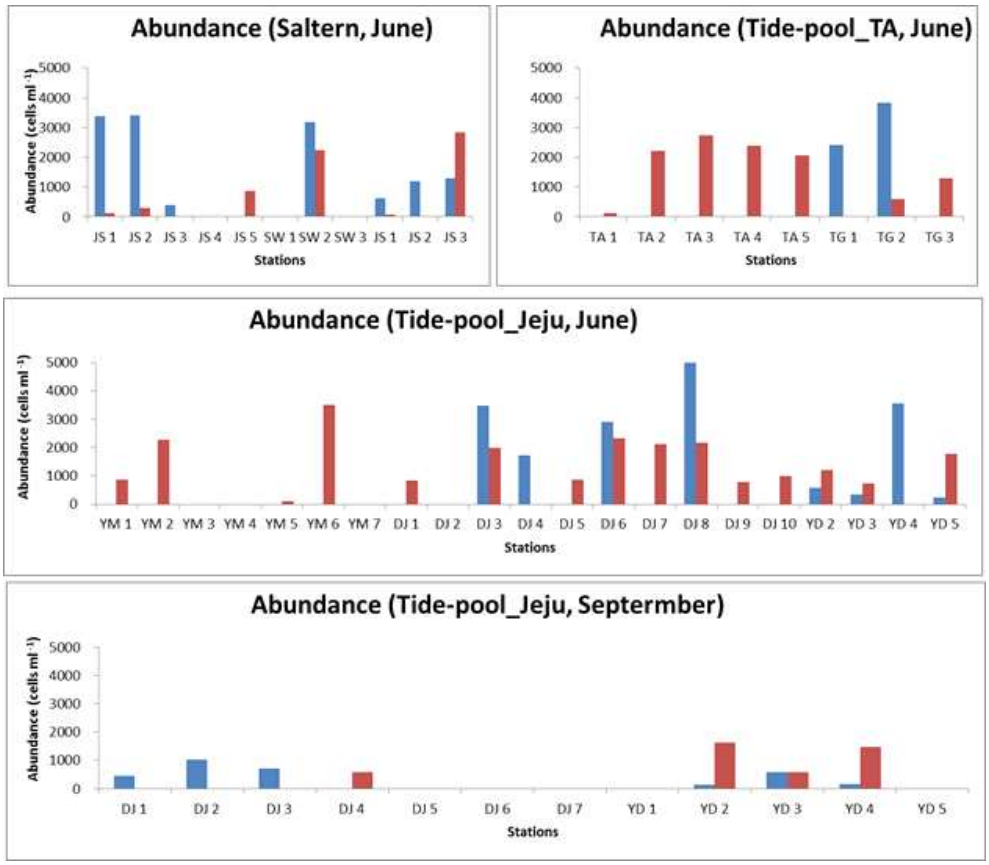

Fig. 4-10. The abundances of *O. marina* and *O. maritima* collected from salterns and tide pools in 2016. (A) Saltern located in Taean (TA) in June, (B) tide pool located in TA in June, (C) tide pool located in Jeju Island in June, (D) tide pool located in Jeju Island in September.

Chapter 5. Characterization of two new compounds from the dinoflagellate *Oxyrrhis marina.*

5.1. Introduction

Microalgae are microscopic plants that contain potential bioactive materials in the form of protei68ns, lipids, glycerols, carotenes, and vitamins (Avagyan, 2008; Priyadarshani and Rath, 2012). Potential bioactive metabolites from microalgae can play a vital role in human health and nutrition. The designing of new functional foods, nutraceuticals, and pharmaceuticals from marine microalgae makes them one of the most valuable marine sources. The use of transgenic microalgae for commercial applications has not yet been reported but holds significant promise (Spolaore et al., 2006). Drug development is the most promising aspect of microalgal biotechnology, although screening of possibilities remains limited (Tramper et al., 2003). Finally, marine microalgae promise to be an important alternative as a future bioenergy source. Heterotrophic protists are known to be important food sources for zooplanktons in the marine environment; they provide essential fatty acids to zooplanktons and act as trophic intermediates between the microbial loop and higher trophic levels (Dolan, 1991; Jeong, 1999; Lowe et al., 2012). Among them, the heterotrophic dinoflagellate *Oxyrrhis marina* has been reported to be a typical efficient producer of long-chain essential fatty acids such as, eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) (Lund et al., 2008; Chu et al., 2008). The unusually and small-sized (15–40 μm) *O. marina* is commonly found in shallow waters

as well as in littoral and supralittoral pools (Lowe et al., 2011a; Roberts et al., 2011). This species is so easy to isolate and culture in the laboratory that it could be used as a model species for a broad range of ecological and biogeographic studies. When investigating the possibility of producing DHA-containing commercial oil through the massive culture of *O. marina,* we recently isolated two types of compounds derived from DHA: a trioxilin (which is a trihydroxy DHA), and a sulfoquinovosyl diacylglycerol (SQDG) with a DHA component. Most trioxilins are hydrolysis products of hepoxilins, which are epoxide derivatives of arachidonic acids (AA, C20:4, n-6) and EPA, metabolized by lipoxygenase (Vogan et al., 2003; Gauthier et al., 2008). It has been reported that trioxilins mediate acetylcholine-induced vasodilation in the rabbit aorta and activate the peroxisome proliferator-activated receptor alfa (PPARα) (Pfister et al., 2003; Yu et al., 2007). Though unusual conversion from DHA to trioxilins in the rat pineal gland and hippocampus has been identified (Reynaud and Pace-Asciak, 1997), this is the first study to report the isolation and the complete chemical structure of a DHA-derived trioxilin from the marine dinoflagellate *O. marina*. The compound showed significant nitric oxide (NO)-inhibitory activity of this trioxilin on lipopolysaccharide (LPS)-induced RAW264.7 cells. Along with the trioxilin, the newly isolated SQDG comprised a glycerol with a docosahexaenoyl substituent. SQDGs have been reported to show inhibitory activity against DNA polymerase, HIV-reverse transcriptase type 1, and platelet-activating factor receptor binding (Ohta et al., 1999; Fan et al., 2008). Like digalactosyldiacylglycerols and other monogalacytosyl analogs isolated from microalgae, the SQDG isolated in our study also showed strong NO-inhibition in murine macrophage RAW264.7 cells (Banskota et al., 2013; Lauritano et al., 2016), more significant than the trioxilin isolated from the same extract. Here we will describe the structure

determination of the two compounds, along with their isolation and biological activity.

I hope that the fundamental as well as applied contributions in this research might serve as potential research and development leads for the benefit of humankind. Microalgae biotechnology will be an important field in the future aimed at the enrichment of targeted algal species, which further establishes a sustainable oceanic environment. The current book is intended to be a handbook for emerging students and experts in the field of biotechnology

5.2. Materials and methods

5.2.1. General

Optical rotations were measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan) with a 5 cm cell. IR spectra were recorded on a JASCO FT/IR 4100 spectrometer. The 1D and 2D NMR spectra were obtained using a Varian VNMRS 500 spectrometer (Palo Alto, CA, USA) in a CD3OD solvent and CDCl3. High-resolution ESI mass spectra were acquired using a Waters SYNAPT G2, courtesy of Korea Basic Science Institute (Ochang Center, Korea). The ESI-MS/MS spectra were obtained in the enhanced product ion scan mode on an ABSCIEX QTRAP 3200 (Foster, CA, USA).

5.2.2. Material (mass cultivation and harvest the cell)

O. marina was isolated from Gunsan, Korea in May 2001, when the water temperature and salinity were 16℃ and 27.7, respectively. Clonal cultures of *O. marina* were fed on dried yeasts (Saccharomyces cerevisiae, Red Star, Lesaffre Yeast Corporation, USA) at 22-24℃ under 10 μE $m^{-2}s^{-1}$of continuous cool white fluorescent light. The yeast (0.1 g/L) was sterilized autoclave at 121℃ for 30 min and then supplied to the predator cells daily. No yeastwas supplied on the last one day to ensure the complete elimination of yeasts from the culture waters.

As the concentration of *O. marina* increased, the culture was transferred to 2 L and 20 L polycarbonate bottles, and then to a 200 L tank and finally to a 1,000 L tank. *O. marina* cells were harvested by centrifugation (15,000 rpm, 10 h) when cell concentration reached with

around 70,000-90,000 cells mL^{-1}. The harvested cells were stored in a deep freezer (-75℃); the moisture was removed using a freeze-dryer (-55℃, 5 mTorr, 24 h).

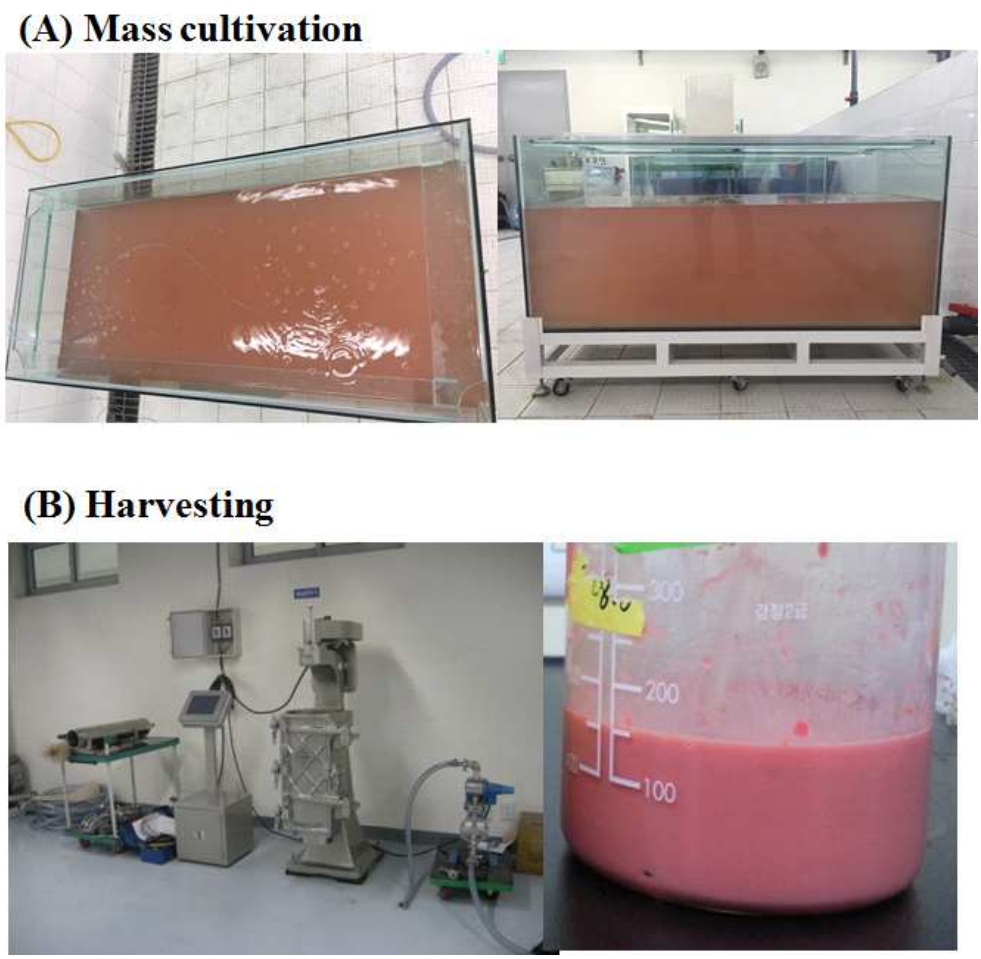

Fig. 5-1. Massive cultivation of *Oxyrrhis marina* (A) and harvesting the culture using by centrifugation (B).

5.2.3. Extraction and Isolation

The freeze-dried cells were extracted with a 100% MeOH solvent to yield 16 g of the extract. The extract was first partitioned into methylene chloride and H_2O, and the organic layer was then repartitioned into 85% MeOH and hexane to eliminate non-polar fatty acids. The aqueous layer was subjected to HPLC with a size exclusion column (100 Å, 300 × 7.8 mm) by eluting the 100% MeOH solvent to generate 10 fractions (Frs 1-10). Fr 6 (35 mg), which was collected at the retention time of 20 min, was purified using reversed-phase HPLC (Synergi polar-RP, 250 mm × 4.6 mm) by gradient elution by changing H_2O:MeOH (v/v) from 60:40 to 100:0 in 35 min, and compound **1** (10 mg) was obtained. Frs 7~ 8 (55 mg) were combined and then purified with the same protocol described above to obtain compound **2** (13 mg).

Compound 1: $[\alpha]_{25}^{D}$ +4.7 (c 1.0, MeOH); IR (film) ν_{max} (cm^{-1}): 3330, 2900, 1716, 1401; ^{1}H and ^{13}C NMR data are given in Table 5-1; HRESI - MS m/z = 377.2325 for $[M - H]^-$ (calcd m/z = 377.2328 for $C_{22}H_{33}O_5$); MS/MS m/z = 112 for $[C_6H_8O_2]^-$, m/z = 137 for $[C_8H_{11}O_3]^-$ − H_2O, and m/z = 181 for $[C_{10}H_{15}O4]^-$ − H_2O.

Compound 2: $[\alpha]_{25}^{D}$ +34.8 (c 0.1, MeOH); IR (film) ν_{max} (cm^{-1}): 3447, 2923, 1736, 1169, 1034; ^{1}H and ^{13}C NMR data are given in Table 5-2; HRESI - MS m/z = 865.5135 for $[M - H]^-$ (calcd m/z = 865.5136 for $C_{47}H_{77}O_{12}S$); MS/MS m/z = 81 for $[SO_3]^-$, m/z = 255 for $[C_6H_{10}O_7S]^-$,

m/z = 537 for $[C_{25}H_{46}O_{11}S]^-$ − H_2O, and m/z = 609 for $[C_{31}H_{46}O_{11}S]^-$ − H_2O.

5.2.4. MTPA Reaction of Reduced Compound 1

Compound 1 was first converted into its methyl ester by reaction with diazomethane in MeOH. After stirring the solution of reduced compound 1 (0.3 mg) and 4-dimethylamino pyridine (30 μL) in $CDCl_3$ (0.1 mL) at 30 °C, R-(−)-MTPA-Cl (5 μL) was added. The reaction progress was monitored by thin-layer chromatography (TLC) on silica gel 4:1 Hex/EtOAc. After ~24 h, the reaction was quenched by the addition of H_2O and dimethyl ether. The organic layer was then concentrated in vacuo. The crude product mixture was eluted with silica-gel Solid Phase Extraction (SPE) using hexane/ethyl acetate (5:1) to produce the S-MTPA-ester reduced 1a as a pale-yellow gum. ^1H NMR ($CDCl_3$, 500 MHz) 5.785 (H-8), 5.774 (H-9), 4.180 (H-7), 4.136 (H-10), 3.669 (H-11), 2.300 (H-6), 2.293 (H-12), 2.168 (H-12); LRESI-MS m/z = 1041 for $[M + H]^+$.

In an entirely analogous way, the R-MTPA-ester reduced 1b was also produced using S-MTPA-Cl. ^1H NMR ($CDCl_3$, 500 MHz) 5.783 (H-8) 5.773 (H-9), 4.179 (H-7), 4.126 (H-10), 3.658 (H-11), 2.315 (H-6), 2.286 (H-12), 2.163 (H-12); LRESI-MS m/z = 1041 for $[M + H]^+$

5.2.5. Cell Cultures

RAW264.7 (mouse macrophage cells), HCT-116 (human colon cancer cells), and HepG2 (human liver carcinoma cells) were obtained from the Korea Cell Line Bank (Seoul, Korea). Neuro-2a (mouse brain neuroblastoma cells) were purchased from ATCC (Manassas, VA, USA).

Cells were maintained in DMEM containing 10% FBS with penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (10 $mg \cdot mL^{-1}$) at 37 °C in a 5% CO_2 atmosphere and 95% relative humidity.

5.2.6. Estimation of NO Production

RAW264.7 macrophage cells were plated in 48-well plates (1 × 105 cells mL^{-1}, 300 μL per well) for 18 h. To remove all the traces of phenol red, the cell culture was washed and the medium was replaced with fresh medium without phenol red. Cells were treated with the sample to be tested for 1 h before exposure to 100 $ng \cdot mL^{-1}$ of LPS. After 24 h of incubation, the nitrite content in the culture media was measured using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). The sample aliquots (100 μL) were mixed with 100 μL of the Griess reagent in a 96-well plate and incubated at room temperature for 10 min. The absorbance at 550 nm was then measured on a microplate reader, and the concentrations were determined using a nitrite standard curve (Napolitano et al., 2009).

5.2.7. Estimation of Cytotoxicity

Compounds to be tested were dissolved in dimethyl sulfoxide (DMSO) (final concentration of 0.1%) and diluted in serum-free culture medium. Before the assay, the cells were seeded and incubated for 24 h in 48-well plates (HCT-116: 1×10^5 cells mL−1; neuro-2a cells: 2×10^5 cells mL^{-1}; HepG2 cell: 5×10^4 cells mL^{-1}; 100 μL per well). The cells were then treated with the vehicle or the compounds at concentrations

indicated for 48 h. The inhibitory activity of each compound on cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with 2 mg mL^{-1} of MTT for 2 h. The supernatant was then aspirated, and 200 μL of DMSO was added to dissolve the formazan. After all the crystals were completely dissolved, the absorbance at 450 nm was measured on a microplate reader. The data were expressed as percentage of viable cells relative to vehicle-treated control cultures. The data were expressed as the means of three independent experiments. IC_{50} values refer to concentration at which 50% inhibition was achieved, and were calculated from regression lines with at least five different concentrations.

5.3. Results and Discussion

The heterotrophic dinoflagellate *O. marina* was cultivated to a volume of 1,000 L by feeding on dried yeasts in sea water. The MeOH extract from the harvested cells was fractionated using HPLC with a size exclusion column, and the fractions were purified by reversed-phase HPLC to generate compounds **1** and **2** (Figure 5-2). Both the compounds were analyzed using a combination of NMR and MS data. Additionally, for determining the stereochemical structure of compound **1**, it was subjected to a chemical reaction.

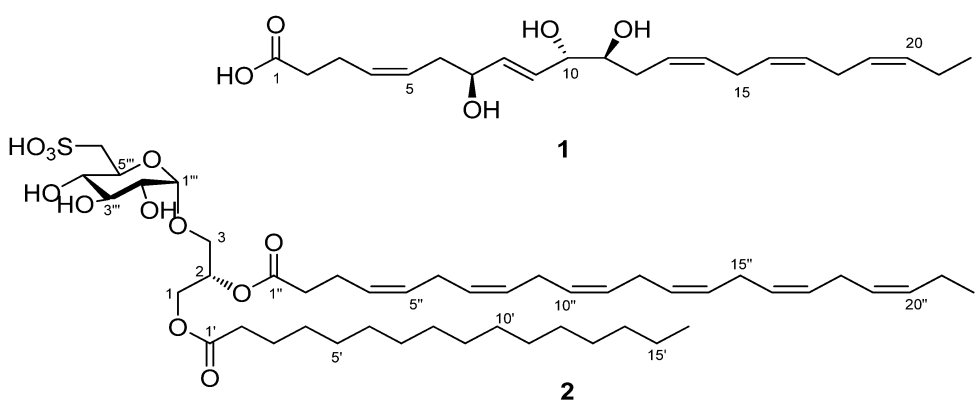

Fig. 5-2. Chemical structure of compounds 1 and 2

Compound **1** was isolated in the form of an amorphous gum. Based on the negative ion peak of $[M - H]^+$ at m/z = 377.2325 (D - 0.3 mmu) from the high resolution ESI mass spectrum, the molecular formula of **1** was determined to be $C_{22}H_{34}O_5$,indicating six degrees of unsaturation. The IR spectrum showed absorption bands corresponding to the hydroxyl (3329 cm^{-1}) and carbonyl (1716 cm^{-1}) groups. The ^{13}CNMR and HSQC-DEPT spectraof compound **1** revealed seven methylenes, three oxymethines, and one methyl group. Apart from this, one carbonyl carbon was also detected from the HMBC spectrum. The molecular structure was elucidated by interpreting the 1D and 2D NMR spectra (Table 5-1). First, the two partial structures shown in bold lines in Figure 2 were constructed using the COSY correlations. One terminal (C-6) in the hydroxylated chain unit was connected with an allyl group from the HMBC correlations of H-7/C-5, H-6/C-4, H-6/C-5, and H-4/C-3. The other HMBC correlations of H-3/C-2 and H-3/C-1 showed a connection between the allylic carbon (C-3) and one methylene, and the termination with a carboxylic acid. On the other hand, the chemical shifts of the remaining four olefinic and two methylene signals suggested a linear structure with methylene-mediated double bonds. This unit might be placed in between the two partial structures, which was confirmed by the TOCSY correlations with the methylene protons at 2.81 and 2.84 ppm. The $D^{4,5}$, $D^{13,14}$, $D^{16,17}$, and $D^{19,20}$ were found to be *cis* forms, based on the chemical shifts of their allylic carbons. In contrast, $D^{8,9}$ was found to be *trans* form, based on the large coupling constant ($^3J_{HH}$=15.7Hz)oftheolefinicprotons.Accordingly,compound**1** was determined to be a new trioxilin, named as (4*Z*, 8*E*, 13*Z*, 16*Z*, 19*Z*)-7, 10, 11-trihydroxydocosapentaenoic acid. This structure was verified by the fragment ion peaks in the negative ESI-MS/MS spectrum as shown in

Figure S1: m/z = 112 for $[C_6H_8O_2]^-$,m/z = 137 for $[C_8H_{11}O_3]^-$ H_2O, and m/z = 181 for $[C_{10}H_{15}O_4]^-$ H_2O.

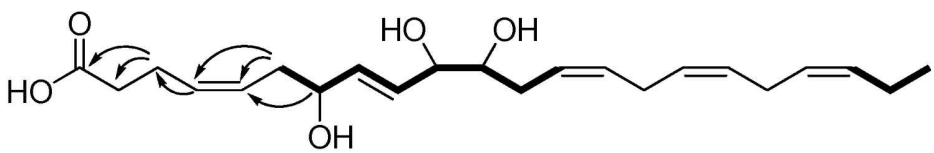

Fig. 5-3. Key COSY (bold lines) and HMBC (arrows) correlations of compound **1**.

Table 5-1. NMR spectral data for compound **1** in CD_3OD.

Position	δ_C	δ_H(*J* in Hz)
1	177.3, C	
2	35.2, CH_2	2.31, m
3	24.2, CH_2	2.33, m
4	131.1, CH	5.46, m
5	127.6, CH	5.47, m
6	36.3, CH_2	2.31, m
7	73.0, CH	4.10, dt (6.1, 6.1)
8	136.2, CH	5.72, dd (15.7, 6.1)
9	130.9, CH	5.78, dd (15.7, 6.1)
10	76.0, CH	3.96, dd (6.1, 4.7)
11	75.9, CH	3.53, dt (8.3, 4.7)
12	31.8, CH_2	2.18, dd (14.9, 8.3); 2.33, m
13	127.4, CH	5.49, m
14	130.7, CH	5.42, m
15	26.8, CH_2	2.84, dd (6.6, 6.1)
16	129.0, CH	5.34, m
17	129.5, CH	5.34, m
18	26.4, CH_2	2.81, dd (7.1, 5.9)
19	128.2, CH	5.29, m
20	132.8, CH	5.37, m
21	21.5, CH_2	2.08, q (7.3)
22	14.7, CH_3	0.96, t (7.6)

The stereochemistry of the three chiral centers in **1** was determined using a *J*-based configuration analysis (Banskota et al., 2013) and the modified Mosher's method. For the *J*-based analysis, the homo- and heteronuclear coupling constants were measured from the well-split proton signals and HETLOC spectrum, respectively. The medium homonuclear coupling constant (J_{HH}=4.7Hz) of H-10/H-11 and the medium heteronuclear coupling constant of H-10/C-11 and H-11/C-10 indicated the interconversion between two conformers (Figure S2). Two additional heteronuclear coupling constants of H-10/C-12 and H-11/C-9 indicated a *threo* relationship between C-10 and C-11. In addition, for determining the absolute stereochemistry of C-7, C-10, and C-11 in compound **1**, Mosher's analysis was performed, in which the compound was treated with *R*(-)- and *S*(+)-MTPA chlorides after methylation with TMS-diazomethane. Each product was esterified with three secondary alcohols in compound **1**, which was recognized by the molecular fragment with *m*/*z* = 1041 in the LRESIMS spectrum. The protons near the three chiral centers of the *S* / *R*-MTPA esters were assigned with the aid of the COSY and TOCSY spectra, and then the differences in chemical shifts of the corresponding protons were then calculated. The absolute configurations of both C-10 and C-11 were determined to be *S*, based on the interpretation of the 1,2-diol system by Riguera et al (Lauritano et al., 2016). Finally, Mosher's analysis of C7 also indicated *S* configuration. Compound **1** was thus determined to be (4*Z*, 8*E*, 13*Z*, 16*Z*, 19*Z*)-7(*S*), 10(*S*), 11(*S*)-trihydroxydocosapentaenoic acid, a metabolite biosynthesized through hepoxilin which was derived from DHA by 11-lipoxygenase.

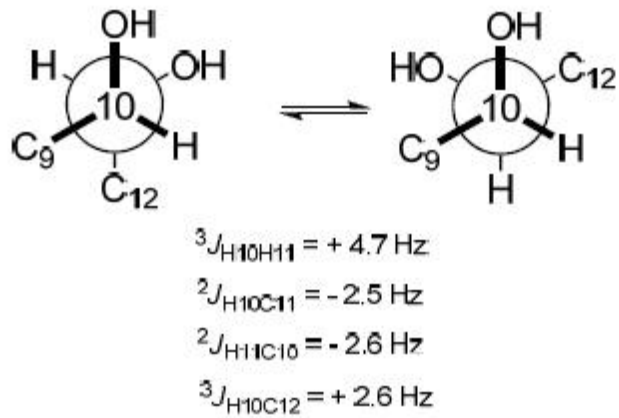

Fig. 5-4. Conformers of C-10 and C-11 of compound **1**, based on J-based configuration analysis.

Compound **2** was isolated as a colorless gum from the same extract. The exact mass of $[M - H]^-$ was measured at *m/z* = 865.5135, and molecular formula to be $C_{47}H_{78}O_{12}S$ (theoretical *m/z* = 865.5136). The ^{1}HNMR spectrum for compound **2** displayed intense aliphatic and olefinic proton signals corresponding to long saturated carbons and polyunsaturated carbons, respectively; it also showed two triplet-methyl protons, which indicated two terminal groups. The ^{13}CNMR spectrum indicated two acyl groups with long carbon chains from two carbonyl carbons, and crowded carbon signals in the ranges of 30~31 ppm and 128~130 ppm. In addition, the presence of a sulfone functional group was inferred from the absorption bands at 1168 and 1034 cm^{-1}in the IR spectrum, and from the molecular formula. Based on this information, the COSY correlations and the proton coupling constants indicated a sulfoquinovose unit. The coupling constant for the anomeric proton was measured to be 3.9 Hz, and was therefore assigned a form. This was consistent with previous reports of the proton chemical shifts and the coupling constants of 6-sulfo-a-*D*-quinovopyransyl group (Gao et al., 2011). Additional HMBC correlations with the two acyl groups suggested the structure of a SQDG. Furthermore, the two fatty acyl groups were determined by MS/MS fragment ions at *m/z* = 537 and 609 as a docosahexanenoyl group and a hexdecanoyl group, respectively (Figure S4). After hydrolysis of compound **2**, a molecular ion peak of $[M - H]^-$ at *m/z* = 255 in the LRESI-MS spectrum confirmed the presence of hexadecanoic acid. The NMR spectral data for protons and carbons in the two acyl groups were assigned using the 2D NMR spectra (Table 2). Specifically, the assignments of H-2′ ~ H-4′ and H-2″ ~ H-4″ were apparently corroborated by the TOCSY correlations of H-2′ and H-2″ which had HMBC correlations with C-1′ and C-1″, respectively. Based on the two acyl groups and the sulfoquinovose, the linkage of the sulfoquinovosyl and

the hexadecanoyl moieties to the sn-1 and sn-3 positions was recognized from the HMBC correlations of H-3/C-1‴ and H-1/C-1′, respectively. Therefore, the structure of compound **2** was determined to be 1-*O*-hexadecanosy-2-*O*-docosahexaenoyl-3-*O*-(6-sulfo-a-*D*-quinovopyranosyl)-glycerol. The configuration of the sn-2 center was determined to be *S* form by comparison of the optical rotation of compound **2** with that reported for other similar compounds (Matsumori et al., 1999; Freire et al., 2005). Compound **2** was found to be a typical SQDG compound, but had a high unsaturated fatty acid (DHA) and a saturated fatty acid.

Compounds **1** and **2** showed antiinflammatory effects on LPS-activated RAW264.7 macrophage cells. The amount of nitrite released into culture media increased 5-fold after exposure to 100 ng/mL of LPS for 24 h. The NO production induced by LPS was significantly suppressed by pretreatment with **1** and **2**, without affecting cell viability; the IC_{50}valuesofcompounds **1** and **2** were calculated to be 22.30 and 10.76 μM, respectively.

The in vitro cytotoxic activity of compounds **1** and **2** against hepatocarcinoma (HepG2), neuroblastoma (Neuro-2a), and colon cancer (HCT-116) cells was also measured. Compounds **1** and **2** did not show any cytotoxicity against any of the three cell lines with a concentration range of 0.1-20 mM. Weak cytotoxicity was observed for compound **1** against Neuro-2a neuroblastoma cells; the relative cell viability was found to be 68~72% at a concentration range of 0.1~20 mM.

Table 5-2. NMR spectral data for compound **2** in CD_3OD.

Position	δ_C	δ_H (J in Hz)
1		4.18, dd (11.0, 6.9); 4.49, dd (11.0, 2.9)
2	64.2, CH_2	5.31, m
3	71.9, CH	3.57, dd (10.8, 6.4); 4.11, dd (10.8, 5.1)
1′	67.1, CH_2	
2′	175.1, C	
3′	35.0, CH_2	2.31, t (7.6)
4′	26.0, CH_2	1.54, q (7.6)
5′ ~ 13′	30.2, CH_2	1.30, m
14′	30.8 ~ 30.5, CH_2	1.30, m
15′	33.1, CH_2	1.26, m
16′	23.7, CH_2	1.30, m
1″	14.4, CH_3	0.89, t (6.9)
2″	174.2, C	
3″	35.1, CH_2	2.39, m
4″	23.7, CH_2	2.39, m
5″, 7″~ 8″, 10″~ 11″, 13″~ 14″, 16″~ 17″, 19″	130.1, CH 129.5~ 129.0, CH_2	5.37, m 5.36, m
6″, 9″ 12″, 15″, 18″	26.6 ~ 26.5, CH_2 132.8, CH	2.85, m
20″	21.5, CH_2	5.36, m
21″	14.7, CH_3	2.08, q (7.6)
22″	100.1, CH	0.96, t (7.6)
1‴	73.5, CH	4.75, d (3.9)
2‴	74.9, CH	3.39, dd (9.8, 3.9)
3‴	75.1, CH	3.62, t (9.8)
4‴	69.9, CH	3.08, dd (9.8, 9.1)
5‴	54.3, CH_2	4.07, td (9.1, 2.0)
6‴		2.91, dd (14.2, 9.1); 3.34, dd (14.2, 2.0)

Chapter 6. Fatty acid composition and docosahexaenoic acid (DHA) content of the heterotrophic dinoflagellate *Oxyrrhis marina* fed on dried yeast: compared with algal prey

6.1. Introduction

Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid that belongs to the omega-3 group (Kitajka et al. 2004, Sijtsma and de Swaaf 2004, Mendes et al. 2009). Some unsaturated fatty acids such as Eicosapentaenoic acid (EPA) and DHA are known to be useful materials for human health (Simopoulos 1991, Arts et al. 2001, Calder and Yaqoob 2009) as they can reduce both coronary heart disease and depression (Joordens et al. 2007). Several protists contain high levels of EPA and / or DHA (Tang and Taal 2005, Burja et al. 2006, Veloza et al. 2006, Liu et al. 2014). In particular, the heterotrophic dinoflagellate *Oxyrrhis marina* is known to produce high levels of DHA when fed on some algal prey (Klein Breteler et al. 1999, Veloza et al. 2006, Adolf et al. 2007, Lund et al. 2008). As large scale culturing of algal prey is both difficult and expensive, a low-cost prey than can be easily cultivated is needed. The fatty acid composition and DHA production of *O. marina* fed on the cryptophyte *Rhodomonas salina* are known to be similar to those fed on the chlorophyte *Dunaliella tertiolecta* (Klein Breteler et al. 1999, Veloza et al. 2006), suggesting that the fatty acid composition and DHA production of *O. marina* may not be dependent on its prey. Therefore, if the fatty acid composition and DHA

production of *O. marina* fed on an easily cultivated low-cost prey are similar or higher than in *O. marina* fed on conventional algal prey species, we can replace the latter prey with the former. *O. marina* is known to feed on diverse prey such as bacteria, nano- or micro-flagellates, phototrophic dinoflagellates, heterotrophic nanoflagellates, ciliates, and yeast (Droop 1959, Jeong et al. 2001, 2003, 2007, 2008, 2010, 2014, Roberts et al. 2011, Yang et al. 2011, Lee et al. 2014). While there have been several studies exploring the lipid content, fatty acid composition, and / or DHA production of *O. marina* fed on algal prey (Klein Breteler et al. 1999, Veloza et al. 2006, Park et al. 2016), no studies have examined *O. marina* fed on heterotrophic protist prey or yeast. As obtaining commercial dried yeast is much easier and cost effective than cultivating algal prey (Coutteau et al. 1992) or heterotrophic protist prey, it is worth investigating the fatty acid composition and / or DHA production of *O. marina* fed on yeast.

In the present study, we explored the fatty acid composition and DHA content of *O. marina* fed on dried yeast and compared the results with *O. marina* fed on *Amphidinium carterae*, which has a high DHA content, and powder of the chlorophyte *Chlorella* sp., which does not contain DHA.

The results of this study provide a basis for understanding the fatty acid composition and DHA production of *O. marina* fed on yeast and algal prey items and suggest a new method for cultivating *O. marina* with high DHA levels using low-cost prey that can be easily obtainable.

6.2. Materials and Methods

6.2.1. Preparation of experimental organisms

A. carterae was grown in enriched f/2 medium without silicate at 20°C and 30 salinity under a 14 : 10 h light : dark cycle of 20 μE $m^{-2}s^{-1}$ provided with the whitecool fluorescent light. Dried *Chlorella* sp. powder was obtained from GapDang Food (Seoul, Korea) and dried yeast (*Saccharomyces cerevisiae*) from Red Star, Lesaffre Yeast Corporation (Milwaukee, WI, USA) (Table 6-1).

O. marina which contained high amount of oil in its body (Fig. 6-1) was isolated from a coastal water off Gunsan, Korea in May 2001 when the water temperature and salinity were 16°C and 27.7, respectively. Clonal cultures of *O. marina* were maintained using *A. carterae* as prey at 20°C under 10 μE m^{-2} s^{-1} of white cool fluorescent continuous light. Cultures of *O. marina* fed on target prey item for 3-4 weeks were used for the analyses of fatty acid composition and DHA when fed on each prey.

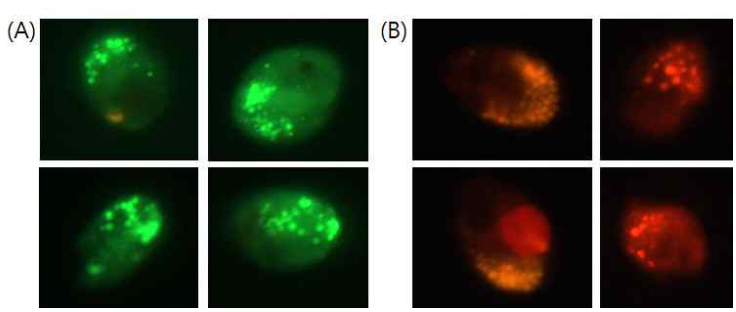

Fig. 6-1. Oil drops from *O. marina* under the fluorescence microscope staining with Bodipy (A) and Nile Red (B).

6.2.2. Analysis of fatty acid composition and DHA content

The experiment was designed to investigate fatty acid composition and DHA content of *O. marina* fed on each of the three different prey items at the same biomass. A dense culture of *O. marina* fed with *A. carterae* was starved for 4-5 d before distributing to triplicate 10-L polycarbonate (PC) bottles. Two liters of *A. carterae* culture at a concentration of ca. 200,000 cells mL^{-1} were added to each PC bottle containing 3L *O. marina* culture, resulting in a final *O. marina* concentration in each bottle ca. 1,800 cells mL^{-1}. These bottles were capped, placed on a shelf, and incubated at 20°C under a continuous illumination of 10 μE $m^{-2}s^{-1}$. After 3 and 6d, 2L of *A. carterae* culture at a concentration of ca. 200,000 cells mL^{-1} was added as prey to each bottle. At 7d, 4L aliquots were taken from each bottle and filtered with GF/C filters (47mm in diameter; Whatman, Kent, UK). At 7 d, most *O. marina* cells were satiated with *A. carterae* so prey cells were not detected in the ambient waters (i.e., close to satiated condition). However, at 11 d, ingested prey cells inside the protoplasm of *O. marina* cells were undetectable that they were in (i.e., close to starved condition). The filters were dried in the freeze dryer overnight. *O. marina* cells incubated with dried *Chlorella* sp. powder or dried yeast were obtained in the same manner described here for *A. carterae* prey.

The weight of each filter containing *O. marina* fed on one of the three different prey items was measured using a microbalance (precision of 0.0001; EL 204-IC; Mettler-Toledo, Columbus, OH, USA) with a blank filter used as a control. Extraction and quantification of lipids were conducted according to Bligh and Dyer (1959); each of the filters was placed in one 1-L glass bottle and then 300 mL chloroform-methanol (2 : 1, v/v) was added. After 2-3 h sonication, 100 mL methanol and 180 mL

water were added to the bottle to make a final solvent ratio of chloroform : methanol : water of 1 : 1 : 0.9. The lipid-chloroform layer in the bottle was separated from the methanol-water layer using a separatory funnel and then placed in a pear-shaped flask. The chloroform was evaporated in a water bath at 32°C using a rotary evaporator (Eyela N-1100; Eyela-Tokyo Rikaki-Kai Co., Tokyo, Japan). The weight of the crude oils left behind (i.e., the total lipid content) was measured gravimetrically using a microbalance. Trans esterification reactions where crude oils are converted to fatty acids were conducted using the modified one-step procedure (methanolic HCl for 2 h at 70°C) of Sukhija and Palmquist (1988).

Fatty acid composition and DHA production were also analyzed using gas chromatography (Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA). The compounds were identified in the NIST mass spectral database and quantified by comparing the peak area with that of the standard (Sigma 47885-U, Supelco 37 component FAME Mix; Supelco, Bellefonte, PA, USA).

Table 6-1. Strains used in this study

Strains	**Date**	**Area**	**Temperature (°C)**	**Salinity (‰)**	**Prey or culture media**	**Culture temperature (°C)**
Oxyrrhis marina	May 2001	Gunsan, Korea	16.0	27.7	*Amphidinium carterae*	20
Amphidinium carterae	NA	USA	NA	NA	f/2-Si medium	20
Chlorella sp. powder	GapDang Food, Seoul, Korea					
Dried yeast	*Saccharomyces cerevisiae*, Red Star, Lesaffre Yeast Corporation, Milwaukee, WI, USA					

NA, not available.

6.3. Results and Discussion

6.3.1. Fatty acid compositions of three prey items and *Oxyrrhis marina* fed on each prey

The fatty acid compositions of yeast, *A. carterae*, and *Chlorella* sp. powder differed from one another (Table 2, Fig. 1); the sequences of the amounts of the fatty acids in the prey species were C18:1 (23.9%) > C16:1 (21.4%) > C16:0 (21.0%) > C18:0 (16.5%) > C18:2 (15.7%) for yeast, C20:0 (22.4%) > C16:0 (22.1%) > C18:4 (19.1%) > C18:1 (4.6%) > C20:1 (4.5%) for *A. carterae*, and C18:2 (49.4%) > C17:0 (24.1%) > C16:0 (13.9%) > C16:1 (4.3%) for *Chlorella* sp. powder (Fig. 1A-C).

Table 6-2. Fatty acid percentage (%) of dried yeast, *Amphidinium carterae,* and *Chlorella* sp. powder that were used as prey items for *Oxyrrhis marina*

	Dried yeast	*A. carterae*	*Chlorella* sp. powder
C10:0	-	-	-
C11:0	-	-	-
C12:0	-	-	-
C13:0	-	-	-
C14:0	-	-	0.9
C14:1	-	-	0.7
C15:0	1.4	-	1.0
C15:1	-	-	-
C16:0	21.0	22.1	13.9
C16:1	21.4	4.1	4.3
C17:0	-	-	24.1
C17:1	-	-	1.4
C18:0	16.5	3.2	-
C18:1c&t	23.9	4.6	2.2
C18:2c&t	15.7	-	49.4
C18:3-n6	-	-	2.2
C18:3-n3	-	-	-
C18:4	-	19.1	-
C20:0	-	2.7	-
C20:1	-	4.5	-
C20:2	-	-	-
C20:5	-	-	-
C22:0	-	22.4	-
C22:1	-	-	-
C23:0	-	-	-
C22:6	**-**	**17.4**	**-**
Total	100	100	100

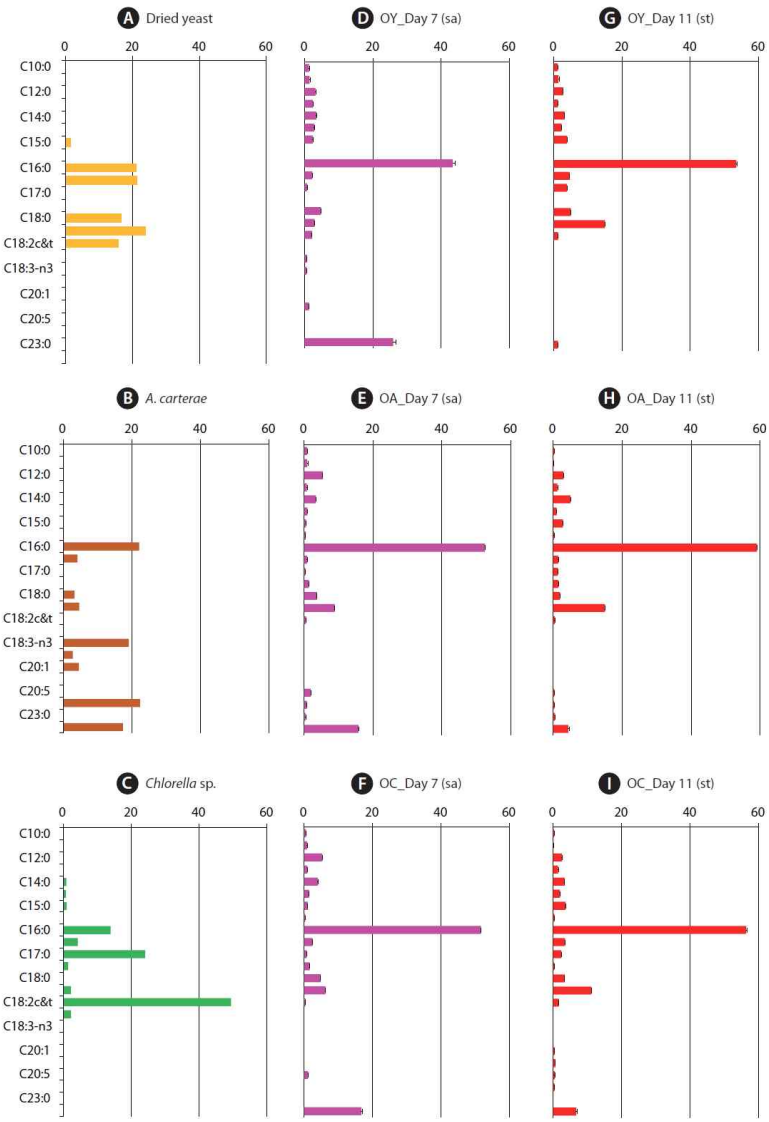

Fig. 6-2. Fatty acid composition as a percentage of total fatty acid methyl ester components from prey items, dried yeast (A), *Amphidinium carterae* (B), and *Chlorella* sp. powder (C) and from *Oxyrrhis marina* fed on each of the three prey species at Day 7 under satiated (sa) conditions (D-F) and at Day 11 under starved (st) condition (G-I). OY, O. marina fed on dried yeast; OA, *O. marina* fed on *A. carterae;* OC, *O. marina* fed on *Chlorella* sp. powder. The error bars represent the standard deviation from the mean of replicate data (n = 2).

The fatty acid compositions of *O. marina* fed on dried yeast, *A. carterae*, and *Chlorella* sp. powder at Day 7 were similar to one another (Table 3, Fig. 1). In particular, the order of the top five fatty acids in *O. marina* fed on these three prey items at Day 7 were the same; the most abundant fatty acid was C16:0 (range = 43.4-52.5% of total fatty acids), and C22:6 (15.7-25.9%), C18:1c&t (2.8-8.9%), C12 (3.1-5.4%), and C18:0 (3.7-4.8%) followed it (Fig. 1D-F). Meanwhile, at Day 11, the most abundant fatty acid was C16:0 (range = 53.5-59.1% of total fatty acids), and C18:1 (11.1-15.0%), C22:6 (1.2-6.7%), C18:0 (2.0-5.0%) and C16:1 (1.5-4.6%) followed it (Fig. 1G-I).

O. marina fed on yeast had only 2.8% C18:1 at Day 7, although the yeast had 23.9% C18:1 and C16:1. Furthermore, *O. marina* fed on yeast, which does not produce DHA, had 25.9% DHA (Fig. 1A, D). At Day 7, *O. marina* fed on *A. carterae* did not have C18:4 or C22:0 (behenic acid) even though the prey contained and (Fig. 1 B, E). Moreover, at Day 7, *O. marina* fed on *Chlorella* sp. powder had only 0.4% C18:2 (linoleic acid) and 0.7% C17:0 (margaric acid) even though the prey had 49.4% C18:2 and 24.1% C17:0 (Fig. 1C & F). At Day 11, the proportion of DHA was dramatically decreased after the prey was completely digested (Fig. 2). *O. marina* fed on yeast, *A. carterae* and *Chlorella* sp. powder had 15.7-25.9% DHA at Day 7 (satiated condition), but the proportion of DHA was decreased to 1.2-6.7% at Day 11 (starved condition, Fig. 2 A-C). In particular, when *O. marina* fed on yeast, 95% of DHA was reduced at Day 11 (Fig. 2A).

This study clearly shows that the fatty acid composition and DHA content of *O. marina* fed on dried yeast were similar to those fed on algal prey, even though the fatty acid composition and DHA content of the three

different prey items differed considerably. This suggests that yeast can be used for efficient DHA production in *O. marina*. In general, heterotrophs have a higher lipid content and higher quality fatty acid composition (i.e., EPA and DHA) than microalgae (Tang and Taal 2005, Burja et al. 2006, Veloza et al. 2006, Liu et al. 2014). However, cultivating the heterotrophs is more difficult and more expensive than cultivating microalgae because of the prey required by the heterotrophs. The cost of obtaining commercial dried yeast (approximately 10 US dollars per kg) is much lower than cultivating cost of general microalgal prey such as *A. carterae* (approximately 160 USD per kg). Yeast, therefore, is an excellent prey for DHA-producing *O. marina*, as it is more easily cultivated and cheaper than the conventional algal prey.

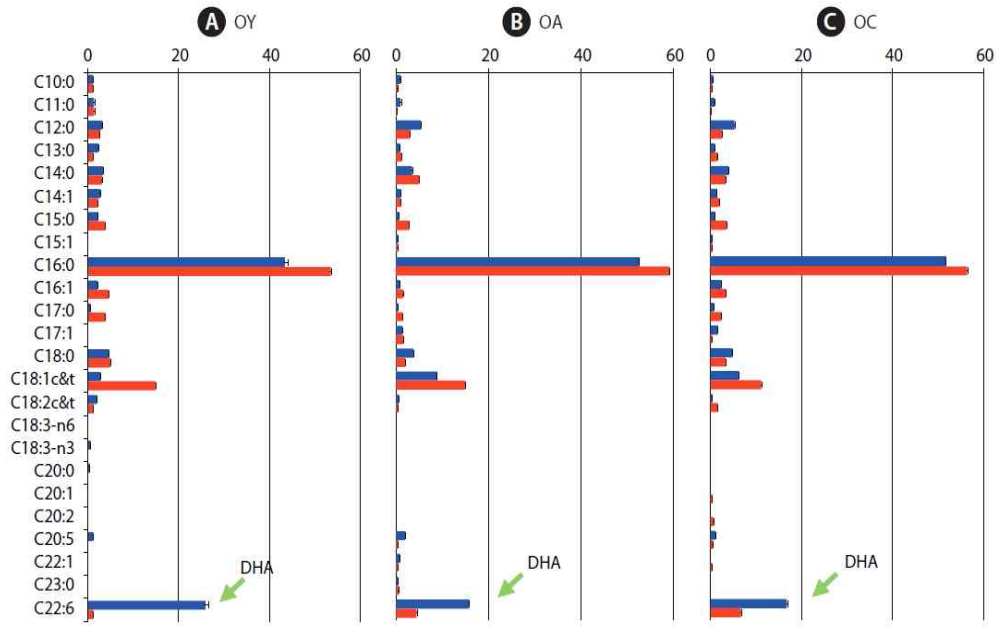

Fig. 6-3. Comparison of fatty acid composition as a percentage of total fatty acid methyl ester components from *Oxyrrhis marina* fed on the three different prey items at Day 7 (blue, satiated condition) and Day 11 (red, starved condition). (A) *O. marina* fed on dried yeast (OY). (B) *O. marina* fed on *A. carterae* (OA). (C) *O. marina* fed on Chlorella sp. powder (OC). The arrow indicates docosahexenoic acid (DHA). The error bars represent the standard deviation from the mean of replicate data (n = 2).

6.3.2. Total lipid and DHA content

The total lipid content (mean ± SD) of dried yeast (17.3± 2.4 μg mg^{-1}) was much lower than that of *A. carterae* (141.8 ± 5.5 μg mg^{-1}) or *Chlorella* sp. powder (41.9 ± 2.6 μg mg^{-1}) (Fig. 3A). However, the total lipid content (±SD) of *O. marina* fed on dried yeast (329.5 ± 5.9 μg mg^{-1}) was approximately 10-30% lower than that of *O. marina* fed on *Chlorella* sp. powder (423.3 ± 52.6 μg mg^{-1}) or *A. carterae* (360.1 ± 43.1 μg mg^{-1}) (Fig. 3B).

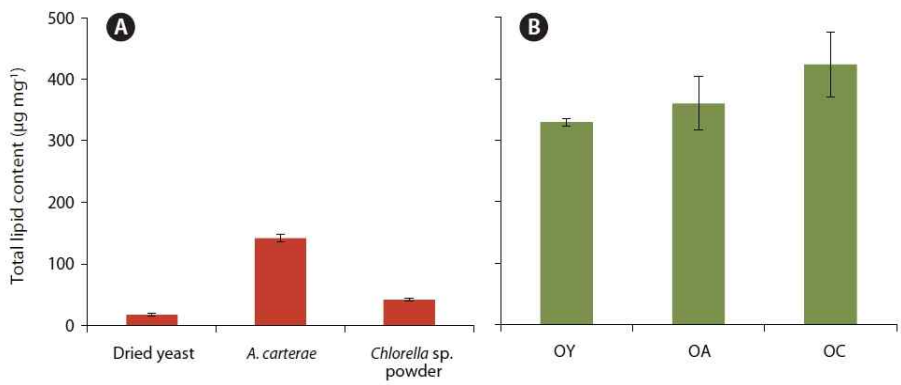

Fig. 6-4. Total lipid content (μg mg^{-1}) of the three prey items, dried yeast, *Amphidinium carterae,* and *Chlorella* sp. powder (A) and *Oxyrrhis marina* fed on each prey, *O. marina* on dried yeast (OY), *A. carterae* (OA), and *Chlorella* sp. powder (OC) (B). The error bar represents the standard deviation from the mean of triplicate data (n = 3).

The lipid content per cell of yeast-fed *O. marina* at Day 11 (starved conditions) was different from that at Day 7 (satiated conditions) (Fig. 4A); the DHA content of yeast-fed *O. marina* at Day 11 (0.90 pg per cell, 1.2%) was much lower than that at Day 7 (52.40 pg per cell, 25.9%); the DHA content of *A. carterae*-fed *O. marina* at Day 11 (3.78 pg per cell, 4.4%) was also much lower than that at Day 7 (26.91 pg per cell, 15.7%); and the DHA content of *Chlorella* sp.-fed *O. marina* fed at Day 11 (4.91 pg per cell, 6.7%) was much lower than that at Day 7 (21.24 pg per cell, 16.7%) (Fig. 4B). As the DHA content of *O. marina* satiated with yeast was greater at Day 7 than at Day 11, harvesting *O. marina* cells fed on dried yeast every 7 d result in higher DHA production than with longer harvesting intervals in this experiments.

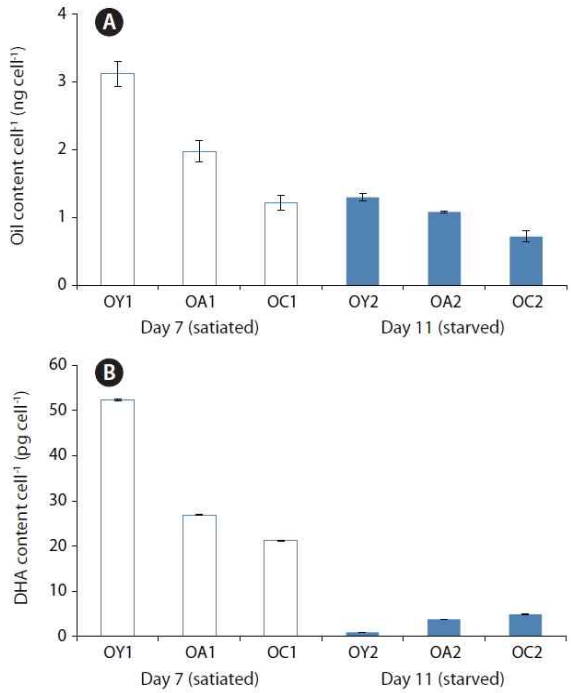

Fig. 6-5. Total lipid content (μg mg^{-1}) of the three prey items, dried yeast, *Amphidinium carterae*, and *Chlorella* sp. powder (A) and *Oxyrrhis marina* fed on each prey, *O. marina* on dried yeast (OY), *A. carterae* (OA), and *Chlorella* sp. powder (OC) (B). The error bar represents the standard deviation from the mean of triplicate data (n = 3).

Table 6-3. Comparison of the fatty acid composition of heterotrophic dinoflagellates *Oxyrrhis marina* fed with three different prey items

FAME	*O. marina* on yeast		*O. marina* on *A. carterae*		*O. marina* on *Chlorella* sp. powder	
	Day 7(satiated)	Day 11(starved)	Day 7(satiated)	Day 11(starved)	Day 7(satiated)	Day 11(starved)
C10:0	1.15 ± 0.30	1.13 ± 0.06	0.88 ± 0.06	0.27 ± 0.12	0.50 ± 0.03	0.27 ± 0.02
C11:0	1.34 ± 0.38	1.35 ± 0.45	0.86 ± 0.59	0.18 ± 0.07	0.81 ± 0.19	0.12 ± 0.03
C12:0	3.09 ± 0.18	2.70 ± 0.03	5.39 ± 0.03	2.96 ± 0.13	5.26 ± 0.18	2.50 ± 0.12
C13:0	2.31 ± 0.10	1.16 ± 0.03	0.80 ± 0.21	1.20 ± 0.16	0.96 ± 0.16	1.41 ± 0.18
C14:0	3.33 ± 0.25	3.14 ± 0.06	3.45 ± 0.17	5.02 ± 0.09	3.99 ± 0.08	3.31 ± 0.09
C14:1	2.69 ± 0.11	2.25 ± 0.04	0.95 ± 0.13	1.02 ± 0.09	1.43 ± 0.01	1.91 ± 0.03
C15:0	2.26 ± 0.10	3.89 ± 0.06	0.54 ± 0.01	2.84 ± 0.07	1.03 ± 0.01	3.55 ± 0.06
C15:1	-	-	0.41 ± 0.02	0.21 ± 0.18	0.33 ± 0.01	0.32 ± 0.01
C16:0	43.38 ± 1.42	53.49 ± 0.44	52.50 ± 0.03	59.08 ± 0.09	51.70 ± 0.03	56.35 ± 0.34
C16:1	2.15 ± 0.07	4.62 ± 0.03	0.80 ± 0.19	1.47 ± 0.06	2.39 ± 0.01	3.40 ± 0.04
C17:0	0.62 ± 0.02	3.85 ± 0.00	0.33 ± 0.02	1.37 ± 0.00	0.73 ± 0.01	2.35 ± 0.00
C17:1	-	-	1.34 ± 0.02	1.51 ± 0.02	1.60 ± 0.00	0.24 ± 0.00
C18:0	4.67 ± 0.06	5.00 ± 0.04	3.71 ± 0.06	1.95 ± 0.07	4.79 ± 0.03	3.30 ± 0.04
C18:1c&t	2.81 ± 0.03	14.98 ± 0.09	8.86 ± 0.05	14.94 ± 0.07	6.25 ± 0.03	11.14 ± 0.13
C18:2c&t	2.05 ± 0.02	1.25 ± 0.01	0.53 ± 0.00	0.46 ± 0.01	0.39 ± 0.00	1.62 ± 0.02
C18:3-n6	-	-	-	-	-	-
C18:3-n3	0.61 ± 0.01	-	-	-	-	-

C20:0	0.44 ± 0.01	-	-	-	-	-
C20:1	-	-	-	-	-	0.39 ± 0.02
C20:2	-	-	-	-	-	0.64 ± 0.02
C20:5	1.20 ± 0.03	-	1.95 ± 0.02	0.33 ± 0.01	1.10±0.03	0.41 ± 0.29
C22:1	-	-	0.79 ± 0.03	0.32 ± 0.01	-	0.32 ± 0.09
C23:0	-	-	0.36 ± 0.26	0.51 ± 0.01	-	-
C22:6&C24:1	25.91 ± 0.63	1.18 ± 0.12	15.74 ± 0.15	4.37 ± 0.64	16.73 ± 0.64	6.67 ± 0.51
Total	**100**	**100**	**100**	**100**	**100**	**100**

It fed on dried yeast powder, *Amphidinium carterae*, and dried *Chlorella* sp. powder. Measurements were taken when the cells were satiated (Day 7) and starved (Day 11). The errors represent the standard deviation from the mean of replicate data (n = 2).

Chapter 7. Overall conclusion

This study has done much to characterize the species *Oxyrrhis marina* and *Oxyrrhis maritima* in the coastal waters of Korea. (1) I investigated the taxonomy of *O. marina* and *O. maritima* isolated from Korea coastal waters, tide-pools, and saltern. (2) I measured the effect of various physiological factors, such as water temperature, salinity, and light intensity, on growth rates in the laboratory. (3) I explored the abundance of *O. marina* and *O. maritima* using real time PCR (qPCR) and digital PCR (dPCR) in the coastal waters of Korea from January to December in 2016. (4) Attempts were also made to derive methods to commercialize useful materials from algal species. In one trial, I isolated two useful bioactive compounds via large-scale cultivation of *O. marina* following and also set up a large-scale culturing system for the production of DHA.

Oxyrrhis marina is a type species of the genus *Oxyrrhis* and has been used as a model species for a broad range of ecological, biological, and evolutionary studies. However, the taxonomy and classification of *Oxyrrhis* species are still vague and controversial, and the other species, *O. maritima* has not been well investigated to date. For this reason, I established six different strains of *O. marina*, which were isolated from the coastal waters off Gunsan (GS), Masan (MS), Shiwha (SH), and Karorim (KRR), Korea, and two strains of *O. maritima* isolated from littoral tidepool waters in Jeju Island, Korea. I confirmed *O. maritima* from JJ and HD were highly divergent from *O. marina* (Clade Ⅰ and Ⅱ)

The environmental factors affecting the growth of *O. marina* and *O. maritima* have been measured in laboratory—*O. marina* did not grow at water temperatures ≥ 35℃, but *O. maritima* did. Thus, the highest temperature for the positive growth of *O. marina* is likely to be around 32℃, while that for *O. marina* is 32℃. I also measured growth rates under various salinity conditions. *O. marina* did not grow at a salinity under 4. As salinity increased from 5 to 50, the growth rate of *O. marina* increased continuously, but it rapidly decreased over 70. However, *O. maritima* species could grow at a salinity of 2. As salinity increased from 3 to 50, the growth rate of *O. marina* increased continuously, but it rapidly decreased as salinity was increased over 70. Thus, *O. marina* and *O. maritima* may have different eco-physiology, reflecting their habitats; *O. maritima* inhabits areas of higher salinity and light intensity (tidal pools) than does *O. marina* (water columns).

I measured the temporal variations in abundance of *O. marina* and *O. maritima* in the coastal waters, tide-pools, and salterns in Korea by qPCR and dPCR. I designed species-specific primers and a probe for the TaqMan assay. I analyzed the gene size of *O. marina* and *O. maritima* using dPCR. The copy number of ITS fragments per cell is unique to each species, and thus can be used to determine the abundance of *O. marina* and *O. maritima* from a laboratory culture. Environmental data supported the laboratory experiment, which indicated that *O. maritima* have more effective growth rates under extreme temperature and salinity conditions.

In the present study, I also tried to find a way to commercialize useful materials from algal species. Two types of bioactive compounds were isolated from a massive culture of *O. marina* in this study: trioxilin and SQDG. Their complete structures were determined using NMR spectroscopy and chemical reactions. The trioxilin was found to be derived from DHA, and the polar lipid SQDG was found to contain a docosahexaenoyl substituent. Compounds 1 and 2 significantly suppressed the NO production induced by LPS in RAW264.7 cells without affecting cell viability.

This study demonstrated that the fatty acid composition and DHA content of *O. marina* fed on dried yeast were comparable to those of *O. marina* fed on algal prey (*A. carterae* or dried powders of *Chlorella* sp.) despite the fact that the fatty acid composition and DHA content of the three different prey items differed considerably. Thus, dried yeast, which is both easily obtainable and cheap, is an excellent prey for DHA production in *O. marina*. The DHA content of *O. marina* was the greatest when they were satiated, suggesting that DHA production may be higher when harvesting *O. marina* cells satiated with dried yeast than when harvesting starved *O. marina*

I hope my research will help understand the role of *Oxyrrhis* species in marine waters and also provide a way to commercialize useful materials from algal species.

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국문초록

종속영양성 와편모류 *Oxyrrhis* 속은 전세계적으로 널리 분포하며 연안, 조간대 및 염전과 같은 다양한 해양환경에 분포하고 있으며 오랜 기간 동안 생태학 및 산업적 중요성 때문에 많은 관심을 받고 있다. 생태학적으로는 후생동물과 타 원생동물의 피식자일 뿐 아니라 박테리아, 나노 플랑크톤, 식물플랑크톤, 혼합영양성 와편모류 등의 포식자로써 먹이망에서도 중요한 역할을 한다. 또한, DHA, EPA, 항염증제 등과 같은 유용한 물질을 생산한다.

Oxyrrhis 속은 1800년대 후반부터 연구가 되기 시작하였으며 한 때 4종주 (*Oxyrrhis marina, O. maritima, O. phaeocysticola, O. tentaculifer*a)가 확립되기도 했으나, 그 이후에 다른 속으로 옮겨진 *P. phaeocysticola* 를 제외한 나머지 3종주 (*Oxyrrhis marina, O. maritima, O. tentaculifer*a)는 *O. marina*로 통합되어졌다. 그동안 유전적 변이가 크다고만 여겨졌던 *O. marina*에 대한 추가적인 연구를 진행한 결과 분자생물학적 차이에 근거하여 1개 이상의 종주가 존재한다는 사실이 밝혀지며 Lowe et al. (2011)에 의해 *O. marina*와 *O. maritima* 두 종으로 재확립 되었지만 형태학적인 차이만으로는 종을 구분하는데 한계가 있다. 두 종의 생태 생리학적 특성에 대한 추가적인 연구는 두 종이 다른 종임을 증명할 수 있는 근거를 제시할 수 있을 것으로 판단되었다. *O. marina*에 대한 생태 생리학적 많은 연구는 기존에 많이 되어져 있었지만, *O. maritima*에 대한 연구는 현재까지 보고된 바 없다. 따라서, 본 연구에서는 한국연안에서 분리된 *O. marina*와 *O. maritima*를 이용하여 정확한 종 동정을 바탕으로 생태 생리학적 연구를 수행했다.

단종 배양체가 확보된 4 strains의 *O. marina* 종주의 SSU rDNA

부분의 염기서열을 비교한 결과 0.3-3.5% 차이를 나타냈으며, *O. maritima* 2 strains 사이의 차이는 0.3%에 불과했다. 그러나, *O. marina*와 *O. maritima*의 SSU rDNA 염기서열의 차이는 17.3 - 18.1% 로서 종간 변이가 큰 것을 나타냈다. 또한, SSU rDNA 및 ITS1-5.8S-ITS2 부분의 DNA를 바탕으로 계통수 분석결과 군산, 마산, 시화, 가로림에서 분리된 *O. marina* 종은 Lineage I (Clade 1과 2)에 속하고, *O. maritima* 종은 Lineage II (Clade 4)와 같은 분지에 포함되었다. 따라서 본 연구에서는 두 종주가 유전학적으로 차이가 난다는 것을 확인했을 뿐 아니라 한국연안에서 분리된 *O. marina* 종은 2개의 계통군 (Clade 1과 2) 으로 나뉘는 것 또한 확인했다.

*O. marina*와 *O. maritima*의 생리학적 특성을 비교하기 위해 다양한 구간의 수온 (5-36℃), 염분 (2-90), 및 광도 (0-100 μE $m^{-2}s^{-1}$)에 따른 종주별 생존가능 한계 및 환경 조건에 따른 성장률을 측정했다. 온도에 따른 성장률을 측정한 결과 *O. marina*는 35℃ 이상에서 생존하지 못하는 반면 *O. maritima*는 생존이 가능했다. 또한, *O. marina*의 최적성장 온도는 25 ℃인 반면 *O. maritima*는 30 ℃ 로 두 종간 차이를 나타냈다. 추가적으로, 생존 가능한 염도를 연구한 결과 *O. marina*는 4보다 낮은 염도에서는 생존하지 못한 반면 *O. maritima*는 2 이상에서 생존 가능했다. 또한, 염도 50에서의 성장률은 *O. marina*가 $0.58d^{1}$로서 *O. maritima*의 $0.67d^{-1}$ 보다 낮았다. 본 연구 결과는 조간대에 서식하는 *O. maritima*가 고염 및 고온에서 수층에서 서식하고 있는 *O. marina*보다 넓은 범위의 환경에서 생존이 가능하다는 결과를 도출할 수 있으며 이는 종주별 서식지 환경에 따른 차이라고 판단한다.

본 연구에서는 *O. marina*와 *O. maritima*의 2016년 1월, 3월, 5월, 7월, 10월 및 12월의 한국연안의 29지역의 항구 및 2016년 6월과 9월에 조간대 (태안 및 제주) 및 염전 (태안)의 현존량을 real-time PCR

(qPCR) 및 digital PCR (dPCR) 기법을 사용하여 분석했다. *O. maritima*의 세포크기가 *O. marina*보다 작지만, gene copy 수는 세포 당 177 ± 9.2 copies로서 *O. marina* 148 ± 6.9 copies 보다 높다. 현장샘플의 현존량을 분석한 결과, 한국연안의 수층에서 *O. maritima*는 4번 출현한 *O. marina* 보다 빈번히 나타났다. 또한, 조간대나 염전의 최대밀도는 (*O. marina* 7,490 cells ml^{-1}, *O. maritima* 3,700 cells ml^{-1}), 수층연안의 최대밀도 (*O. marina* 39 cells ml^{-1}, *O. maritima* 3 cells ml^{-1}) 보다 상대적으로 월등히 높았다.

*O. marina*는 성장률이 빠를 뿐 아니라 몸속에 고함량의 지질을 포함시킨다. 추출된 지질에서 NMR 및 화학반응을 통하여 구조분석결과 트리옥실린 (trioxilin)과 sulfoquinovosyl diacylglycerol (SQDG)의 두 개의 신규화합물을 분리했다. 화합물 1은 트리옥실린으로서 (4Z,8E,13Z,16Z,19Z) -7(S),10(S),11(S) -trihydroxydocosapentaenoic acid를, SQDG는 (2S)-1-O- hexadecanosy -2-O -docosahexaenoyl -3-O-(6-sulfo-α-d-quinovopyranosyl)-glycerol의 구조를 가진다. 두 화합물 모두 DHA로부터 유래되었으며, RAW 264.7 세포에서 산화질소 억제반응을 나타냈다. 화합물 1과 2는 세포 생존능력에 영향을 주지 않으면서 RAW264.7 세포에서 LPS에 의해 유도된 NO 생성을 유의하게 억제 하였다. 화합물 1과 2에 대해서 간암 (HepG2), 신경모세포종 (Neuro-2a), 대장암 (HCT-116) 세포에 대한 억제 반응을 분석한 결과 화합물2에서는 세포독성을 나타내지 않았으나 화합물 2에서는 신경모세포종을 약하게 억제하는 것으로 관찰되었다.

*O. marina*종주를 상업화하기 위해 저비용 고효율로 배양하는 것에 대한 연구를 진행했다. *O. marina*는 조류먹이를 섭식 후 고함량의 DHA를 몸속에 축적하지만 조류먹이를 배양하기 위해서는 고비용이 필요하다. 하지만, 경제성을 위해서는 배양이 용이하며 저비용의 배양법을 탐색

해야했다. 본 연구에서 *O. marina* 종주의 3종의 먹이 (*A. carterae,* 클로렐라 분말, 건조효모)에 따른 지방산 및 DHA의 함량을 비교했다. *O. marina*의 DHA 함량 (전체 지방산 중 비율)은 건조효모로 포화되었을 때 세포당 52.40 pg (25.9 %)로서 *A. carterae* (세포당 26.91 pg; 15.7%) 또는 *Chlorella* sp. 분말 (세포 당 21.24 pg; 16.7 %)보다 높았다. 또한, 먹이를 섭식 후 소화되지 않은 상태 (satiated condition)가 먹이가 소화된 상태 (starved condition) 보다 높은 DHA 함량을 나타냈다. 먹이 1kg 당 비용은 건조효모가 10 USD로써 *A. carterae* (약 160 USD) 보다 저렴하다. 따라서, 고효율 저비용의 *O. marina*를 이용한 DHA 생산을 위해서는 건조효모를 먹이로 제공 후 소화되지 않은 상태에서 수확하는 것이다. 본 연구에서는 유용물질의 원재료를 대량생산하기 위해 톤 단위의 새로운 배양시스템을 성공적으로 수립했다.

본 연구에서는 한국연안에 출현하고 있는 *Oxyrrhis* 속의 단종배양체 확보를 통해 정확한 종 동정을 실시했다. 형태학적·분자생물학적 특성을 바탕으로 정확한 종동정을 실시했으며, 수온, 염분 및 광도 등의 다양한 환경 변수에 따른 성장률에 대한 실험을 실험실내에서 진행했다. 현장에서의 다양한 환경변수에 따른 현존량을 새로운 기법인 qPCR과 dPCR을 통해 분석했다. 실험실내 실험 결과와 현장 자료를 비교함으로써 두 종주는 처음 출현했던 서식지 환경과 관련에 있다는 결론을 얻었다. 또한, *O. marina*로부터 유용한 신규화합물을 분리하는데 성공하였으며, 효율적인 DHA 생산을 위해 대량배양 방법을 개발했다. 이 연구결과는 해양생태계에서 *Oxyrrhis* 속의 생태 생리학적 역할을 잘 이해하고 해양 플랑크톤으로부터 유용한 물질을 상업화하는데 기여할 것으로 사료된다.

주요어 : 종속영양성 와편모류, *Oxyrrhis*, 분류학, 생태학, 현존량, 유용 신물질, DHA 생산

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